Université de Montréal

Is GALA solution (DuraGraft®) the Optimal Preservation Solution to Protect the Endothelial Function of Saphenous Vein Grafts used in Coronary Artery Bypass Grafting surgery?

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Ce mémoire intitulé :

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Résumé

Est-ce que la Solution de Préservation Intra-opératoire GALA (DuraGraft®) est Optimale pour Protéger les Fonctions Endothéliales des Greffons de Veines Saphènes pour la Chirurgie de Pontages Aortocoronariens ?

INTRODUCTION : Les greffons de veine saphène interne (GVS) sont encore régulièrement utilisés comme conduits en chirurgie de pontage aorto-coronarien (PAC). Les dommages subis par les segments de veine saphène pendant le prélèvement et le stockage favorisent une dysfonction endothéliale qui se manifeste par une diminution de la production d'oxyde nitrique et/ou par une augmentation du niveau de stress oxydant pouvant entraîner une défaillance du greffon veineux se traduisant par une occlusion. La solution saline héparinée est la solution de préservation de référence malgré plusieurs études démontrant ses effets néfastes sur les GVS. GALA est une solution de préservation de greffons autologues vasculaires spécialement développée pour préserver l'intégrité structurale et fonctionnelle de la couche endothéliale des greffons utilisés en chirurgie de pontages aorto-coronariens.

OBJECTIF : Comparer la préservation de l'intégrité des fonctions endothéliales des greffons de veine saphène après le stockage dans la solution GALA versus dans la solution saline héparinée dans le cadre d'une étude contrôlée et randomisée en étudiant la réactivité vasculaire en chambres d'organes.

RÉSULTATS : Les segments de GVS d'un total de quinze patients ont été obtenus et divisés en anneaux de 3 mm de largeur. Il n'y avait pas de différences significatives dans les niveaux de contraction en réponse au chlorure de potassium, à la phényléphrine, ni dans les concentrations de phényléphrine nécessaires pour atteindre le niveau de contraction cible entre les anneaux du groupe GALA versus le groupe de saline héparinée. Les courbes dose-réponse du groupe solution GALA ont démontré une amélioration significative des relaxations dépendantes de l'endothélium par rapport au groupe solution saline héparinée. Les contractions et relaxations indépendantes de l'endothélium induites respectivement par la phényléphrine et le nitroprussiate de sodium étaient similaires dans les anneaux de GVS des deux groupes.

CONCLUSION : L'utilisation intra-opératoire d'une solution développée spécifiquement pour la préservation de l'intégrité endothéliales présente un potentiel d'avantages cliniques chez les patients qui subissent une chirurgie de PAC. Les observations précédentes suggèrent que la solution GALA pourrait réduire la dysfonction endothéliale associée à la défaillance des greffons veineux et incite des évaluations à long terme plus approfondies dans le cadre d'essais cliniques.

MOTS-CLÉS : Dysfonction endothéliale, veine saphène humaine, solution de préservation de greffons, chirurgie de pontages aorto-coronarien (PAC), relaxations endothélium-dépendantes, réactivité vasculaire, chambres d'organes.

Abstract

Is GALA solution (DuraGraft®) the Optimal Preservation Solution to Protect the Endothelial Function of Saphenous Vein Grafts used in Coronary Artery Bypass Grafting Surgery?

INTRODUCTION: Saphenous vein grafts (SVGs) are still commonly used as conduits for coronary artery bypass grafting (CABG). Injury to SVGs during harvesting and storage promotes endothelial dysfunction, which is attributed to a decrease in production of nitric oxide and/or increased level of oxidative stress that can lead to vein graft failure (VGF). Heparinized saline is still the standard of care intraoperative preservation solution despite several studies demonstrating its detrimental effects on SVGs. GALA is an innovative one-time intraoperative graft storage solution developed to preserve endothelial integrity.

OBJECTIVE: To investigate, in a randomized controlled study, endothelial functional integrity of saphenous vein grafts following storage in GALA vs heparinized saline using *ex vivo* vascular reactivity studies in organ chamber experiments.

RESULTS: Segments of saphenous vein grafts from a total of fifteen patients were obtained and divided into 3 mm wide rings for evaluation. There were no significant differences in the levels of contraction in response to potassium chloride and to phenylephrine between groups, nor in the concentrations of phenylephrine needed to achieve the target level of contraction in saphenous vein graft rings. Concentration-response curves of the GALA group demonstrated a significant improvement in endothelium-dependent relaxations compared to the heparinized saline group. Endothelium-independent contractions and relaxations induced by phenylephrine and sodium nitroprusside, respectively, were not altered in saphenous vein graft rings from both groups.

CONCLUSIONS: Intraoperative application of a solution developed for graft preservation demonstrated a potential benefit to protect endothelial and vascular functional integrity in saphenous vein grafts of patients undergoing CABG. These data suggest that the GALA solution may reduce endothelial dysfunction associated with vein graft failure and warrant further long-term evaluation in clinical trials.

KEYWORDS: Endothelial dysfunction, human saphenous vein, graft preservation solution, Coronary artery bypass graft (CABG) surgery, endothelium-dependent relaxations, vascular reactivity, organ chambers.

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List of abbreviations

[MCP]-1: Monocyte chemoattractant protein

[PAI]-1: Plasminogen activator inhibitor

·NO: Nitric oxide

·OH: Radical hydroxyl

5-HT: Serotonin

AA: Arachidonic acid

AC: Adenylate cyclase

ACE: Angiotensin converting enzyme

ACh: Acetylcholine

ADMA: Asymmetric dimethylarginine

ADP: Adenosine diphosphate

AGE: Advanced glycation end products

AHB: Autologous heparinized blood

Akt: Alpha-serine/threonine

Ang I: Angiotensin I

Ang II: Angiotensin II

ApoE: Apolipoprotein E

ARBs: Angiotensin receptor blockers

AT_1R: Angiotensin type 1 receptor

AT_2R: Angiotensin type 2 receptor

ATP: Adenosine triphosphate

AVP: Arginine vasopressin

AWB: Autologous whole blood

BB: Beta-blockers

BH_4 : Tetrahydrobiopterin or (6R-)5,6,7,8-tetrahydro-l-biopterin

BK: Bradykinin

BK_{ca}: Ca^{2+}-activated K^+ channel

BMS: Bare-metal stents

BRS: Bioresorbable stents

Ca^{2+}: Calcium

CABG: Coronary artery bypass grafting

CAD: Coronary artery disease

CaM: Calmodulin

CaV-1: Caveolin 1

CaV-3: Caveolin 3

CCh: Carbachol

CEL: Celsior

CGD: Chronic granulomatous disease

cGMP: Cyclic guanosine monophosphate

CMD: Coronary microvascular disease

CO: Carbon monoxide

COX: Cyclooxygenase

CRP: C-reactive protein

DAF: Diaminofluorescein

DAG: Diacylglycerol

DES: Drug-eluting stents

EC: Endothelial cells

EC_{50}: Half-maximal effective concentration

ECE: Endothelin converting enzyme

EDCF: Endothelium-derived contracting factors

EDHF: Endothelium-derived hyperpolarizing factor

EDRF: Endothelium-derived relaxing factors

EET: Epoxyeicosatrienoic

E_{max}: Maximal response

eNOS or type III: endothelial nitric oxide synthase

ET: Endothelin

ET-1: Endothelin-1

ET_A and ET_B: Endothelin receptor A, Endothelin receptor B

ETC: Electron transport chain

FAD: Flavin adenine dinucleotide

FMD: Flow-mediated dilation

FMN: Flavin mononucleotide

GALA: Glutathione, ascorbic acid, L-Arginine

GPCR: G protein-coupled receptor

H_2B: Dihydrobiopterin

H_2O_2: Hydrogen peroxide

BH_4: Tetrahydrobiopterin

HBSS: Hank's balanced salt solution

HLS: Heparinized lidocaine saline

HMG-CoA: Hydroxymethylglutaryl-coenzyme A

Hsp90: Heat shock protein 90

HSV: Human saphenous vein

HSVG: Human saphenous vein graft

IC ACh: Intracoronary acetylcholine

IH: Intimal hyperplasia

IL-1: Interleukin-1

iNOS or type II: Inducible nitric oxide synthase

ITA: Internal thoracic artery

K^+: Potassium

K_{Ca}: K^+ calcium-sensitive channels

KCl: Potassium chloride

LDL: Low-density lipoprotein

L-Arg: L-arginine

L-NAME: N_ω-nitro-L-arginine methyl ester

MACCE: Death, nonfatal myocardial infarction, repeat-revascularisation, or stroke

MACE: Death, nonfatal myocardial infarction, repeat revascularization

MCP-1: Monocyte chemoattractant protein-1

MHI: Montreal Heart Institute

MI: Myocardial infarction

MLCP: Myosin light chain phosphatase

mM: Millimolar

MMP: Matrix-degrading metalloproteinase

NaCl: Normal saline or sodium chlorite

NADPH: Nicotinamide-adenine-dinucleotide phosphate

NF-κB: Nuclear factor-κB

NHE-1: sodium-hydrogen exchanger 1

nNOS or type I: neuronal nitric oxide synthase

mRNA: messenger ribonucleic acid

NOS: Nitric oxide synthase

NOX: NADPH oxidase

NS: Normal saline

$O_2 \cdot^-$: Superoxide Anion

$ONOO^-$: Peroxynitrite

Ox-LDL: Oxidized lipoproteins

PCI: Percutaneous coronary intervention

PE: Phenylephrin

pEC_{50}: Negative logarithm to base 10 of the EC_{50}

$PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$

PGH_2: Prostaglandin H_2

PGI_2: Prostacyclin

PI3K/Akt: Phosphatidylinositol 3-kinase/Protein kinase B

PKG: protein kinase G

PKC: Protein kinase C

PL A: Plasma-Lyte A

PLA2: Phospholipase A2

PLC: Phospholipase C

PMCA: Plasma membrane calcium ATPases

PMVs: Porcine mammary veins

PUFAs: Polyunsaturated fatty acids

RAAS: Renin-angiotensin-aldosterone system

ROS: Reactive oxygen species

SEM: Standard error of the mean

Ser 1177: Serine 1177

sGC: Soluble guanylate cyclase enzyme

SNP: Sodium nitroprusside

SOC: Standard of care

SOD: Superoxide dismutase

SR: Sarcoplasmic reticulum

SVG: Saphenous vein grafts

TCM: Tissue culture medium

TF: Tissue factor

TGF_{β}: Transforming growth factor β

Thr: Thrombin

TNF: Tumor necrosis factor

TP-receptors: Thromboxane-prostanoid receptors

TXA_2: Thromboxane A_2

UM: Unmanipulated

UP: Unprepared

UW: University of Wisconsin

VA: Veterans Affairs

VCAM-1: Vascular cell adhesion protein-1

VDCC: Voltage-dependent Ca^{2+} channels

VEGF: Vascular endothelial growth factor

VGF: Vein graft failure or venous graft failure

VSM: Vascular smooth muscle

VSMC: Vascular smooth muscle cells

vWF: Von Willebrand Factor

XDH: Xanthine dehydrogenase

XO: Xanthine oxidase

XOR: Xanthine oxidoreductase

Dedicated to my parents Touria and Mustapha, my brother Othmane, and my sister Salma for their love, endless support, and encouragement.

Chapter 1 – The endothelium

The endothelium lining the vascular tree of each organ adapts to accommodate its functional requirements and performs additional distinctive biological tasks [1]. Few examples of endothelial organ-specific roles are the unique arrangement of capillaries in alveoli maximizing gas exchange in the lungs [1], myocardial function in the heart, and phagocytosis in the liver and spleen [2]. The endothelial monolayer strategically lies between the lumen of the blood vessel and the vascular smooth muscle composed of endothelial cells (EC) elongated in the direction of blood flow linked by three types of junctions: tight junctions, gap junctions, and *adherens* junction [2, 3]. The endothelium is a selective permeable barrier that allows blood components passage to the tissue and cellular waste products from the tissue to the blood for clearance [3]. Other than the regulation of the vascular tone, the healthy endothelium plays a central role in cellular adhesion, thromboresistance, smooth muscle cell proliferation, immune defense, and minimizing vessel wall inflammation [4]. In cardiovascular conditions such as hypertension and hypercholesterolemia, the pulsatile hydrostatic pressure damages the lining and promotes endothelial dysfunction resulting in proliferation of the underlying smooth muscle and connective tissues cells [1, 5].

Endothelial cells have outer membrane receptors on their surface that detect changes in hemodynamic forces, physical or chemical stimuli and produce endothelium-derived relaxing factors (EDRF) and endothelium–derived contracting factors (EDCF). These factors diffuse into the underlying smooth muscle to generate an acute vascular tone response regulating blood pressure and blood flow [2, 3]. The endothelium is a paracrine, endocrine, autocrine organ central to the maintenance of vascular homeostasis, blood fluidity and regulation of vascular tone [6]. Vasomotion maintains a balance of tissue oxygen supply by regulating the vessel tone and diameter for long-term organ perfusion [4].

1.1-Endothelium-derived relaxing factors (EDRF): Nitric oxide

The endothelium pioneers, Furchgott and Zawadski, made the breakthrough discovery of nitric oxide (·NO) in 1980. During their experiments, they observed relaxation of rabbit aortic rings in response to acetylcholine only with an intact endothelium thus postulating the existence of an

EDRF [2, 3, 7]. It was only in 1987 that the EDRF responsible for these relaxations was identified as ·NO [3].

·NO is a colourless and non-prostanoid, labile, free radical synthesized by the enzyme NO synthase (NOS). There are three different isoforms of NOS: neuronal NOS (nNOS or type I), inducible NOS (iNOS or type II), and endothelial NOS (eNOS or type III). nNOS is constitutively expressed in the brain and plays an important role in the control of neuronal functions and nerve signal transduction [8-10]. This isoform is present in other non-neuronal cells such as in the endothelium, smooth muscle cells of several types of vessels in animals [11]. The iNOS is expressed in almost all types of cells including smooth muscle cells and especially in macrophages and fibroblasts. It thus plays a major role in immune defense as its activation occurs in response to endotoxins, tumor necrosis factor (TNF), bacterial products and other cytokines. [8, 12, 13]. eNOS is mainly expressed in ECs and is localized more precisely in caveolae which are small invaginations of the plasma membrane containing the transmembrane protein caveolin [3]. Like nNOS, eNOS is constitutively expressed and generates low amounts of ·NO produced over few seconds whereas iNOS stimulation is delayed but results in the production of large amounts of ·NO over several hours [8]. Both constitutively expressed eNOS and nNOS isoforms are calcium-dependent and control basal ·NO levels whereas iNOS is insensitive to calcium levels but rather responds to inflammation signals [13].

The NOS enzyme is a homodimer, and each monomer has two domains [13, 14]: An N-terminal oxygenase domain attached to a C-terminal reductase domain via a calmodulin-binding motif [14]. The three isoforms use L-Arginine (L-Arg) and oxygen (O_2) in a stereospecific two-steps oxygen oxidation reaction to produce ·NO and L-Citrulline [13, 14]. NOS requires several cofactors to function. For instance, the co-substrate nicotinamide-adenine-dinucleotide phosphate (NADPH), and the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) have their binding sites in the reductase domain whereas the substrate L-Arginine, the cofactor (6*R*-)5,6,7,8-tetrahydro-l-biopterin (BH_4), and heme binding motifs are located on the oxygenase domain [14].

In order to exert functional catalytic activity, NOS needs to undergo dimerization through the interaction of the heme site of each monomer in the presence of heme [13]. With eNOS and nNOS, ·NO synthesis is activated by the binding of calmodulin (CaM) in response to enhanced calcium (Ca^{2+}) levels (between 200 and 400 nM) conversely to nNOS where calmodulin binds even at low intracellular calcium concentrations (below 40 nM) [15] . CaM is required for proper domain alignment and for enhancement of electron transfer from the reductase to the oxygenase domain [13, 14]. NOS transfers electrons from NADPH via FAD and FMN in the reductase domain to the heme in the oxygenase domain where BH_4, O_2 and L-arginine are bound [15]. The substrate L-Arginine is then oxidized and deaminated to generate ·NO and L-Citrulline via two consecutive monooxygenation reactions [16]. Thus, arginine derived analogs are tools that allow pharmacological manipulations of NOS activity and are often used in clinical research to assess the distribution and role of ·NO [2]. Inhibitors such as the nonselective inhibitor of NOS N_{ω}-nitro-L-arginine methyl ester (L-NAME), act by blocking NOS access to its substrate L-Arginine in a competitive manner [2, 17].

1.1.1 Activation of eNOS

eNOS can be activated by two distinct mechanisms. The less predominant one is eNOS phosphorylation by the protein alpha-serine/threonine (Akt) on the Serine 1177 (Ser 1177) residue. Shear stress is the key eNOS activator since the end goal is to adjust organ perfusion to the cardiac output. Some endogenous signalling molecules also stimulate ·NO production including bradykinin (BK), endothelin-1 (ET-1), adenosine diphosphate (ADP), adenosine triphosphate (ATP), thrombin, vascular endothelial growth factor (VEGF) in response to oxygen deprivation, and serotonin (5-HT) resulting from platelet aggregation [4]. The serotonergic receptors and those for thrombin, and ET-1 are coupled with the pertussis toxin-sensitive G_i proteins, while BK, ATP, and ADP receptors couple to G_q proteins [18, 19]. These molecules are involved in the activation of eNOS by inducing a rise in the levels of cytosolic calcium [19]. Other factors that influence NOS activity include the level of NOS mRNA and protein expression, the amount of asymmetric dimethylarginine (ADMA) which is an endogenous eNOS competitive inhibitor, the amount of cofactors BH_4, and NADPH [20]. Heat shock protein 90 (Hsp90) interacts with eNOS to enhance NO production via two pathways: 1) by stabilization of the $Ca^{2+/}$CaM complex and enhancement of its affinity for eNOS 2) by preventing the dephosphorylation of Akt, the kinase responsible for Ser1177 residue phosphorylation [21]. On the other hand, caveolin membrane proteins caveolins 1

and 3 (CaV-1 and CaV-3), respectively found in endothelial cells and cardiomyocytes, suppress eNOS activity by forming a complex when in the resting state [21]. However, once intracellular calcium rises, the complex Ca^{2+}/CaM acts as an allosteric competitor to the caveolins and stimulates eNOS Ca^{2+}-dependent activation [22].

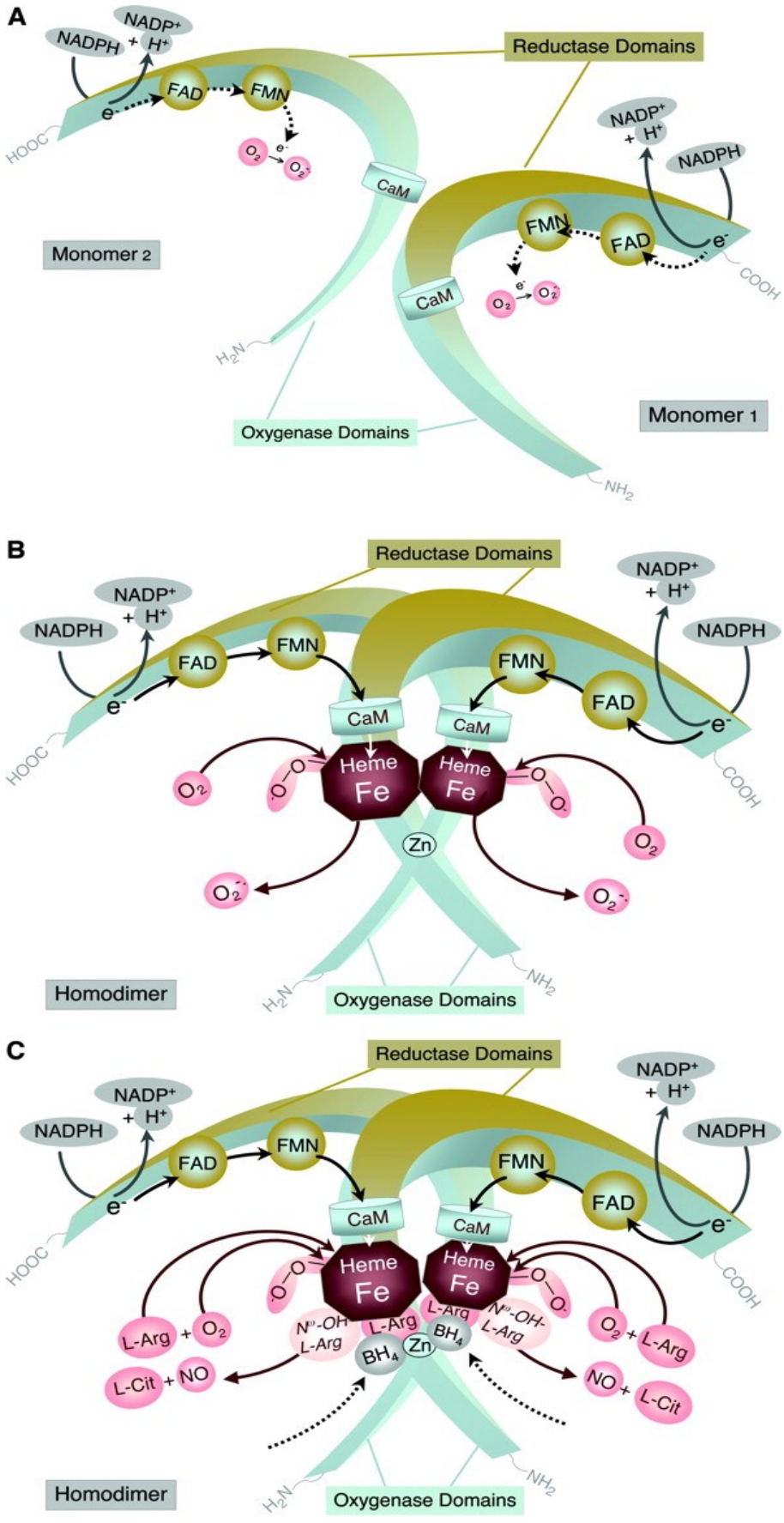
A
NADPH
NADP+ + H+
FAD
FMN
e-
HOOC
O2
O2-
CaM
Reductase Domains
Monomer 2
Monomer 1
H2N
Oxygenase Domains
NH2
COOH
B
Heme Fe
Zn
Homodimer
C
L-Arg + O2
L-Cit + NO
NO + L-Cit
BH4
N(ω)-OH-L-Arg

Figure 1. – eNOS structure and mechanisms of ·NO production

A, The structure of eNOS is made of two monomers. Each subunit contains a reductase and an oxygenase domain. Monomers and isolated reductase domains transfer electrons from NADPH to FAD and FMN to reduce molecular oxygen to $O_2\cdot^-$. Calmodulin (CaM) can also bind to isolated reductase domains of NOS monomers in order to stimulate electron transfer occurring within the said domain. Single monomers cannot bind the cofactor BH_4 or the substrate l-arginine to undergo NO· production. **B**, The attachment of the heme group is required for dimerization and to have the active form of NOS enzyme. The heme group is responsible for the interdomain electron transfer from the flavins to the heme of the opposite monomer and enhances the rates of NADPH oxidation significantly for more effective $O_2\cdot^-$ production. **C,** In the presence of sufficient amounts of the substrate L-arginine and cofactor BH_4, NOS dimers couple their heme and O_2 reduction to synthesize NO· and forming L-Citrulline [23]. *Permission to reuse obtained (Annex 1).*

1.2-Endothelium-dependent relaxation pathways

Since ·NO is gaseous, it easily diffuses through endothelial cell membranes to the underlying smooth muscle cells. ·NO interacts with the iron atom in the heme group to activate soluble guanylate cyclase enzyme (sGC) which drives the production of cyclic guanosine monophosphate (cGMP) [3]. cGMP then activates Protein Kinase G (PKG) which in turn causes smooth muscle relaxation via two dominant pathways: 1) by activating myosin light chain phosphatase (MLCP) which dephosphorylates myosin inducing a conformational change that blocks its interaction with actin causing muscle relaxation. 2) by modifying the conductance of large Ca^{2+}-activated K^+ channel (BK_{ca}) located on the SMC membrane.

The increase in BK_{ca} activity leads to a larger influx of the K^+ cation into SMC causing membrane hyperpolarization and closure of voltage-dependent Ca^{2+} channels (VDCC) while inhibiting plasma membrane calcium ATPases (PMCA), therefore decreasing the total Ca^{2+} import into the cell. 3) through phosphorylating phospholamban, a protein that usually inhibits the Ca^{2+} pump SERCA responsible for calcium uptake into the sarcoplasmic reticulum (SR). Deactivation of phospholamban increases SERCA activity and helps sequester Ca^{2+} into the SR thus reducing Ca^{2+} levels into the cytoplasm [24]. ·NO has other crucial protective effects on the vascular wall such as the prevention of smooth muscle cell proliferation, inhibition of platelet aggregation, monocyte adhesion, and expression of adhesion molecules [25].

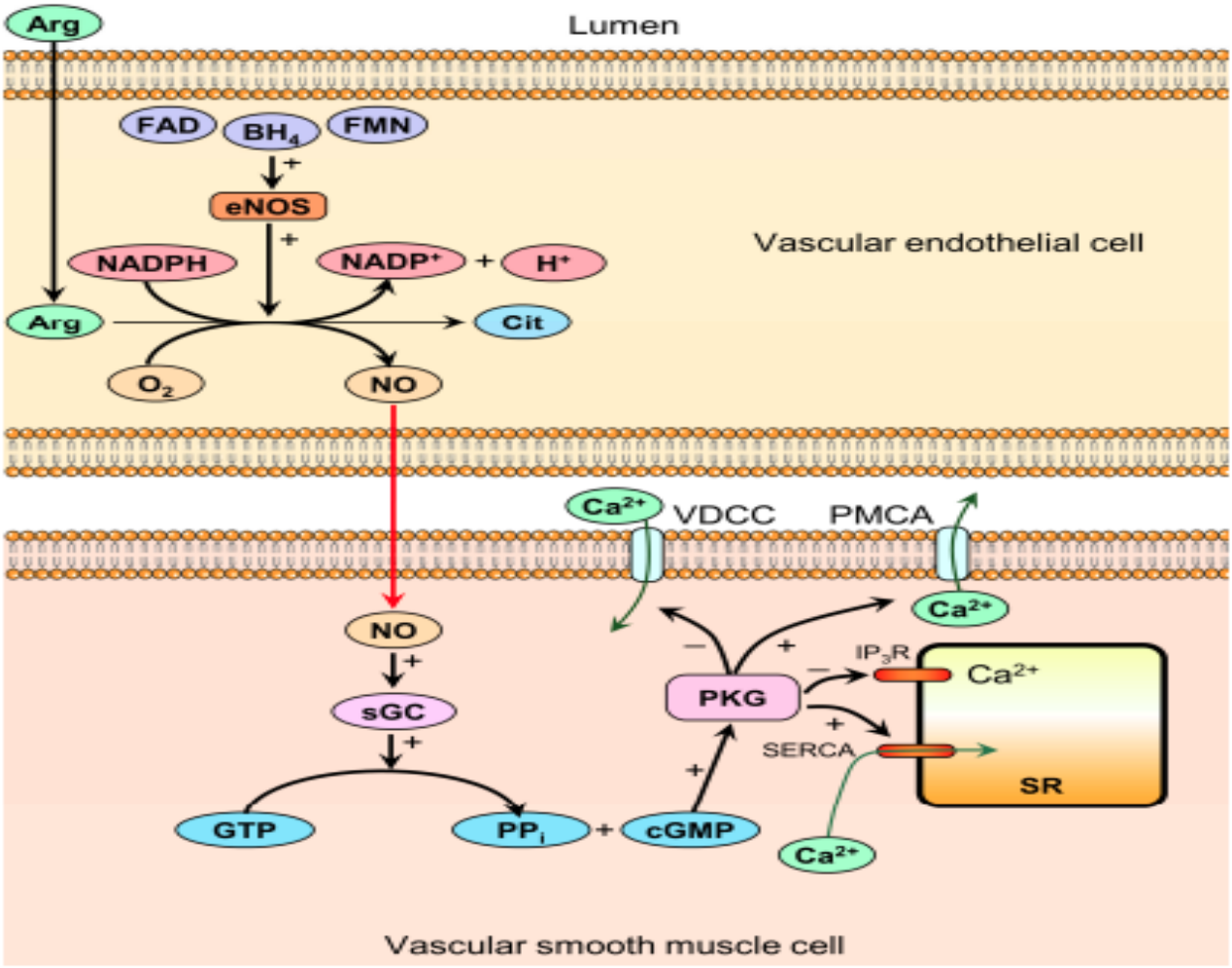

Figure 2. – Mechanisms of nitric oxide-mediated vasodilation

eNOS requires the co-substrate O_2, and cofactors NADPH, BH_4, FAD, FMN to convert the main substrate L-Arg into nitric oxide (NO). NO diffuses from the endothelial cell to the smooth muscle cell and activates (sGC), resulting in cGMP production. Subsequently, PKG is stimulated by cGMP resulting in decreased intracellular Ca^{2+} by 1) Inhibition of voltage-dependent calcium channels (VDCC) and reduction of calcium influx; 2) Stimulation of plasma membrane calcium ATPases (PMCA), increasing ATP-dependent calcium efflux; 3) Activation of SERCA pump, increasing sequestration of calcium from the cytoplasm to the SR. Decreased intracellular Ca^{2+} causes smooth muscle relaxation via the activation of MLCK and the inhibition of MCLP resulting in vasodilation [26]. *Open access article - no permission required (Annex 1).*

1.2-Endothelium-derived relaxing factors (EDRF): Prostacyclins (PGI_2)

Prostacyclin (PGI_2) is another vasodilator produced predominantly in EC but also in the media and adventitia in response to hypoxia [12]. Similar to thromboxane A_2 (TXA_2), these lipid mediators belong to the prostanoid group of eicosanoids. Arachidonic acid (AA) is the precursor of PGI_2 and must be mobilized from the plasma membrane via the enzymatic actions of phospholipase A2 (PLA2) which relies on calcium to become active [27]. AA is then converted to PGI_2 through the enzyme cyclooxygenase (COX) in response to shear stress and to chemical signaling molecules such as bradykinin, acetylcholine and adenine nucleotides [2, 20]. COX enzyme has two isoforms COX1 and COX2 that differ in structure, tissue distribution, and function [28]. COX1 is constitutively expressed and its activity is dependent on intracellular calcium concentrations whereas COX2 is induced upon the occurrence of vascular injury and inflammation [28].

Thromboxane A_2 (TXA_2) is another prostanoid product synthesized in parallel to PGI_2 from the same upstream precursors in both endothelial cells and platelets [27]. In contrast to PGI_2, TXA_2 is a potent vasoconstrictor and promotes platelet aggregation [28]. In most normal arteries, PGI_2 results in vascular smooth muscle relaxation by acting on the IP receptor (G_s-coupled protein) located on the membrane of SMCs. This PGI_2/IP interaction stimulates adenylate cyclase (AC) for the production of the second messenger cyclic adenosine monophosphate (cAMP) which activates protein kinase A (PKA) [28, 29]. Myosin-light chain kinase (MLCK) activity is inhibited by cAMP, therefore myosin cannot be phosphorylated to initiate the usual myosin-actin interaction for cross-bridging resulting in the inhibition of contraction machinery [28]. In addition, cAMP also leads to Ca^{2+} extrusion at the cell surface and SR Ca^{2+} pumps while K^+ channels promote K^+ influx resulting in cell hyperpolarization and subsequent vascular smooth muscle relaxation.

The activation kinetics of PLA_2 relies on the presence of agonists such as BK and adenine nucleotides and requires higher calcium requirement than eNOS. Production of prostanoids is transient making the role of PGI_2 in the maintenance of vascular tone more limited [4, 18] whereas

·NO is continuously released [27]. Moreover, in most vessels, PGI_2 does not contribute in a predominant manner to endothelium-dependent relaxations but its effect is essentially additive to those of ·NO, the main vasodilator as there is cross talk between NO and PGI_2 pathways. For example, ·NO indirectly enhances the action of PGI_2 as the rise in cGMP levels inhibits the enzyme phosphodiesterase III that usually degrades cAMP. The primary roles of PGI_2 are its cardioprotective, antithrombotic, and antiplatelet activities shown through the maintenance of balance between PGI_2 and TXA_2 synthesis due to their opposite effects [30].

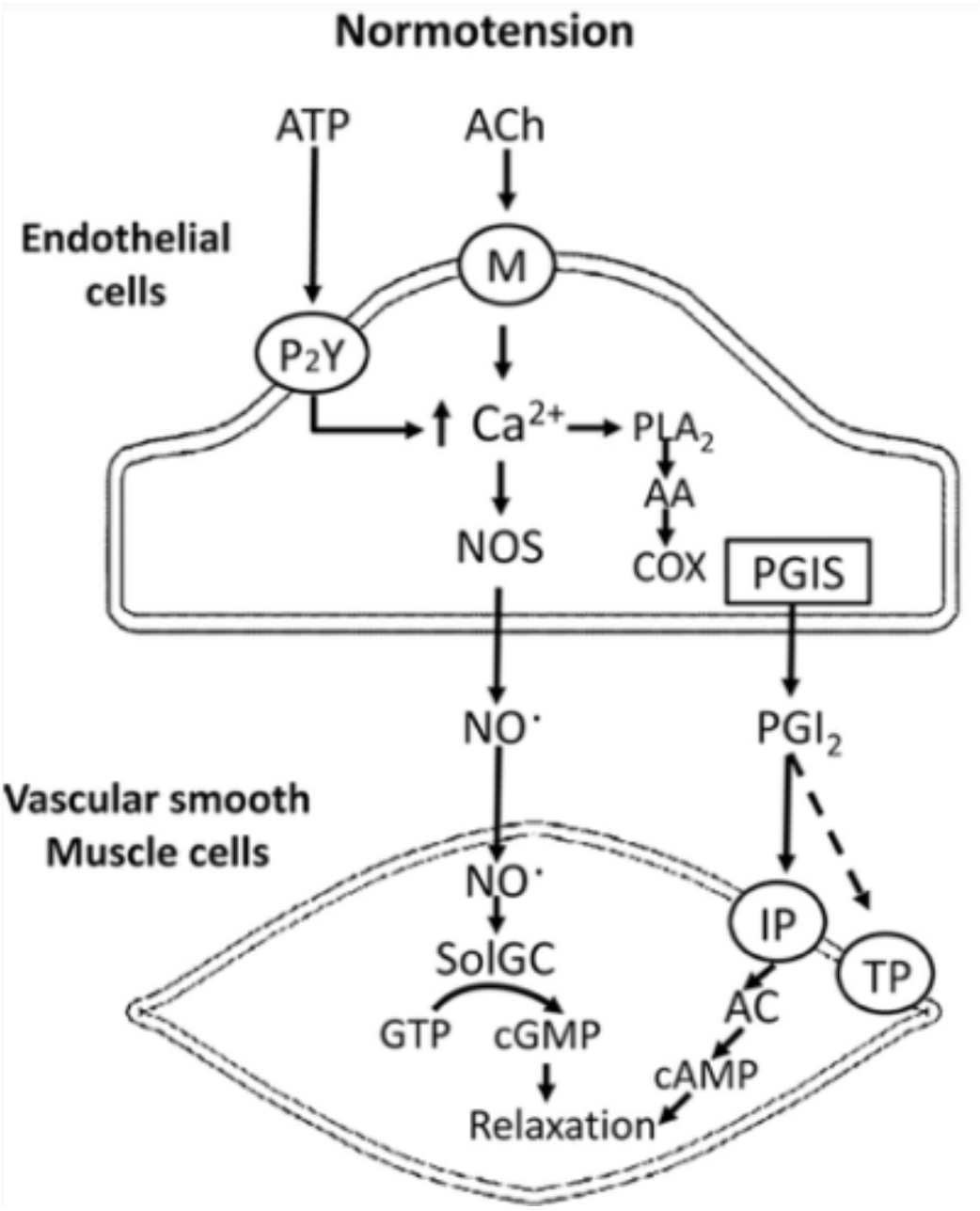

Figure 3. – Endothelium-dependent relaxation effects of ACh in normotensive rat aorta.

ACh stimulates NO production and PGI synthesis from the precursor AA via COX in EC. NO diffuses into SMCs and increases cGMP concentration while PGI_2 acts mainly on IP receptor to increase cAMP concentration causing VSMCs relaxation. Endothelium-dependent relaxations effects from the PGI_2 pathway are additive to the NO, the main vasodilator [31]. *Permission to reuse obtained (Annex 1).*

1.3-Endothelium-derived hyperpolarizing factors (EDHF)

Electrophysiological studies have reported that SMC of resistance and medium blood vessels from various species, including humans, display endothelium-dependent hyperpolarisation even in the presence of COX and NOS inhibitors [25]. In these vascular beds SMCs relaxations are caused by diffusion of endothelium-derived hyperpolarizing factors (EDHF), a set of substances rather than a single diffusible chemical [32]. The molecular basis of EDHFs-mediated vasodilation is extremely complex and speculation remains since the mechanisms of relaxation seem to differ according to the species, race and gender as well as the type and size of blood vessels, [20, 32]. Overall, NO remains the main vasodilator for large conduit vessels whereas EDHF mediates small resistance vessels vasodilation especially in the microcirculation where blood pressure and tissue perfusion are regulated [33]. Although their precise nature is still not fully characterized, several substances have been proposed as EDHF candidates including epoxyeicosatrienoic acids (EETs) derived from AA (metabolites of the arachidonic P450 epoxygenase pathway), carbon monoxide (CO), adenosine, peptides, and H_2O_2 in mouse, in human mesenteric arteries, and in porcine and canine coronary microvessels [33, 34]. Reactive oxygen species (ROS) are usually considered harmful and contribute greatly to the onset and evolution of cardiovascular diseases however EDH/H_2O_2 at physiological levels can play an important role as signaling molecules [33].

EDHFs are synthesized upon agonists stimulation, but also under the influence of greater shear stress [34]. The release of EDHFs synthesized in ECs is induced by an increase in intracellular calcium which activates K^+ calcium-sensitive channels (K_{Ca}) both on EC and SMCs. Hyperpolarization of EC is mediated by the increase in K^+ ion movement and by opening calcium-activated channels BK_{ca}, IK_{ca}, and SK_{ca} according to their respective conductance (big, intermediate, and small conductance) [20], resulting in K^+ efflux and EC endothelium-dependent hyperpolarization [20]. SMCs relaxation is thought to be induced through two general distinct mechanisms: 1) EDHFs are released from EC and diffuse into SMCs to open BK_{ca} channels causing SMCs hyperpolarization [35]; 2) K^+ ions released through the opening of K_{ca} accumulate in the intercellular cleft, leading to the hyperpolarization of SMCs by activating K^+ conductances and/or Na^+/K^+-ATPase [36]. Of significance, there is also direct transmission of EC hyperpolarization to SMCs through gap junction, a phenomenon called “myoendothelial gap junctional”

communication [3, 36]. Thus, the contribution of hyperpolarization to endothelium-dependent vascular relaxation is more significant in resistance vessels or microvessels while remaining essentially additive to ·NO vasodilation in larger size arteries [12]. One probable reason is that the number of heterocellular gap junctions increases as the arterial diameter decreases [3]. However, when ·NO activity decreases or its production is inhibited, EDHF maintains the level of endothelium-dependent relaxations close to normal [12].

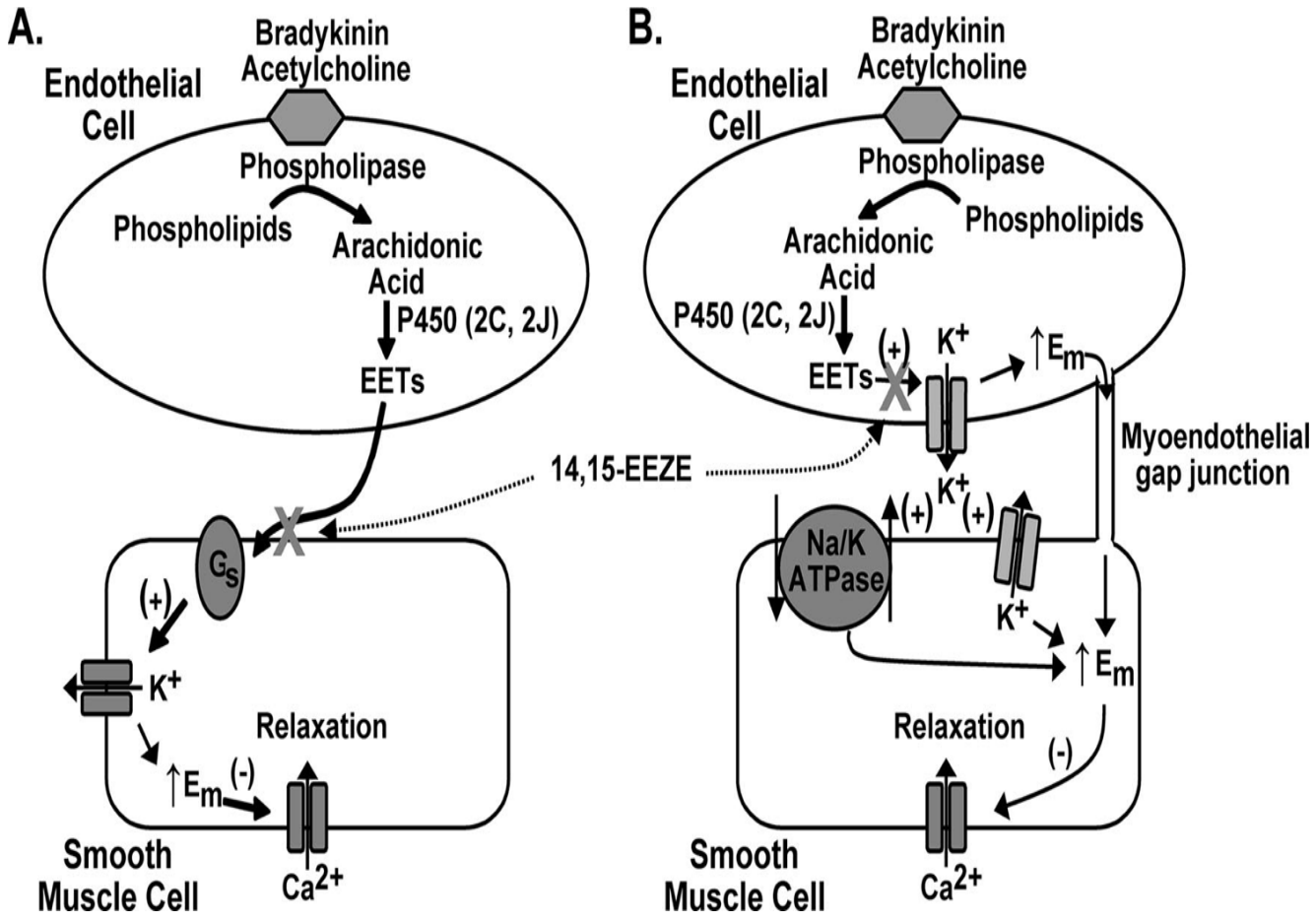

Figure 4. – Mechanisms of SMCs relaxation mediated by the endothelium- derived hyperpolarizing factor EETs

A, EETs diffuse into SMCs and open BK_{ca} channels causing SMCs hyperpolarization **B**, K^+ ions released through the opening of K_{ca} accumulate in intercellular clefts activating K^+ conductance and/or Na^+/K^+-ATPase and the hyperpolarizing SMCs. There is also direct transmission of EC hyperpolarization to SMCs through gap junction called "myoendothelial gap junctional" communication to cause hyperpolarization and relaxation [35]. *Permission to reuse obtained (Annex 1).*

1.4-Endothelium-derived contracting factors (EDCF)

Endothelial control of the vascular tone control is the result of a balance between relaxations and contraction (constriction) of vascular smooth muscle cells. Under given circumstances such as physical forces (stretch, pressure) and hypoxia, the withdrawal of EDRF results into acute increase in SMCs contraction through the release of diffusible naturally occurring substances from EC termed endothelium-derived contracting factors (EDCF). These include vasoconstrictor peptides (angiotensin II, endothelin-1), oxygen-derived free radicals, vasoconstrictors prostanoïds (prostaglandin H_2 (PGH_2), thromboxane A_2 (TXA_2), and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) [37].

1.4.1-Angiotensin II (Ang II)

The renin-angiotensin-aldosterone system (RAAS) is involved in cardiovascular physiology and pathophysiology through the regulation of fluid volume, and blood pressure via angiotensin II (Ang II) and subsequently aldosterone production [3]. Ang II is synthesized through a cascade of enzymatic reactions where the enzyme renin cleaves angiotensinogen into Ang I, then the enzyme ACE (angiotensin converting enzyme) further cleaves Ang I into Ang II [3, 38]. Ang II is a major potent vasoconstrictor that plays an important role in hypertension by stimulating the secretion of aldosterone which promotes sodium retention [3]. ACE are located on the EC surface allowing easy access to cleave Ang I in the plasma [39]. ACE also plays an important role in degradation and inactivation of the potent indirect vasoconstrictor bradykinin (BK) by stimulating the release of NO, EDHF and PGI_2 [3]. Ang I can also be converted to the alternative peptide Ang-(1-7), via a mechanism independent of ACE, through the action of three neutral endopeptidases (NEP): NEP 24.11, NEP 24.15, and NEP 24.26 [40]. Greater understanding of the RAAS was achieved with recent findings showing that Ang 1-7 exerts cardioprotective and antagonistic effects to those of Ang II through the G protein-coupled receptor (GPCR) Mas[40]. An in vivo study of the effect of Ang-1-7/AT1-R axis in mice showed counteraction phenylephrine–induced contraction of abdominal aorta identifying Ang-1-7/AT1-R as potential therapeutic target in hypertension [41].

Ang II can bind to four subclasses of receptors, the two main ones are type 1 (AT_1R) and type 2 (AT_2R). AT_1R mediates the main physiological effects of Ang II and are located in multiple

tissues including vascular smooth muscle, endothelium, cardiac, cerebral, renal and adrenal tissue [42]. Since ECs contain a greater amount of AT_1R receptors than AT_2R, upon stimulation of AT_1R, vasoconstriction is triggered as well as SMC proliferation [43]. Moreover, Ang II directly generates VSM contraction by binding to AT_1R, causing interaction with heterotrimeric G_q proteins which activates phospholipase C (PLC) generating the secondary messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) [44]. IP_3 causes the release of intracellular calcium in addition to opening calcium channels located on the SMC membrane which allows calcium entry. Tension develops as Ca^{2+}/calmodulin dependent MLCK switches on MLC phosphorylation [44]. On the other hand, DAG activates protein kinase C (PKC) which results in myosin light chain phosphatase (MLCP) inhibition and potentiation of myosin phosphorylation to cause myofilament Ca^{2+} sensitization for contraction [44]. Another effect of Ang II binding to AT_1R receptor is the production of ROS such as O_2^- via membrane bound NADH/NADPH oxidase [45]. Several studies have demonstrated that ROS play a pivotal role in diverse pathological conditions such as hypertension, endothelial dysfunction and atherosclerosis [45].

1.4.2-Endothelin (ET)

Endothelin peptides are a family of 21 amino acid chains found in three different isoforms: ET-1, ET-2, and ET-3 [46, 47] and are related to the snake venom safaratoxin [3]. Endothelin was first isolated, purified and sequenced in 1988 and it is one of the most powerful vasoconstrictor ever identified [46, 47]. ET-1, the most abundant and studied form, is unstable and has short half-life [46, 48]. The precursor of endothelins is the 92 amino acids proendothelin peptide converted to endothelins by the endothelin converting enzyme (ECE) [48]. The production of endothelins can be inhibited by ECE metalloprotease inhibitors and can be reduced by ·NO and prostacyclin [48].

Healthy subjects have low levels of circulating ET-1 since up to 75 % of synthesized ET -1 is released from ECs towards SMCs suggesting that ET-1 is not produced in substantial amounts in the absence of pathological stimuli or when its production is effectively inhibited [3, 48]. In fact, ET-1 is released in response to stimuli such as adrenaline, thrombin, ang II, cytokines, in the presence of low shear stress or pulsatile stretch as well as during hypoxia [2]. ET-1 then binds to its two G_q-protein coupled receptors ET_A and ET_B [47]. SMCs mainly express ET_A receptors but also the subtype ET_{b2} of ET_b, receptors but at lower levels. Both types of receptors provoke potent

vascular vasoconstriction [3, 47]. However, stimulation of ET_b receptors subtype ET_{b1} present on endothelial cells results in NO production and the release of PGI_2 and EDHF inducing vasodilation [49-51]. On the other hand, the rise of intracellular calcium influx via L-type calcium channels and activation of phospholipase C (PLC) to produce the second messengers IP_3 and DAG, which mobilize the calcium outflow from intracellular storages in the SR, are the mechanisms by which ET_A SMC receptors stimulation cause vasoconstriction counterbalancing ET_{b1} mediated vasodilation [49]. In addition, DAG activates protein kinase C (PKC) which affects gene transcription via the activation of Ras/Raf/MEK/MAPK cascade increasing VSMCs contraction [49, 52]. The reaction mediated by ET-1/ ET_{b1} triggering EDRF and EDHF release could serve as a feedback mechanism to restore normal vascular tone [3] but high production and/or activity of ET-1 greatly contribute to pathological conditions related to dysfunctional endothelium such as in pulmonary and systemic hypertension, heart failure, and atherosclerosis [3].

1.4.3-Vasconstrictor prostanoids (PGH_2, TXA_2, $PGF_{2\alpha}$)

Prostaglandin H_2 (PGH_2) is the end product of the metabolism of the common precursor of prostaglandins AA in EC by the constitutive enzyme COX1 and inducible enzyme COX2. PGH_2 is a short-lived and biologically active endoperoxide [53] which is then converted by a set of synthases into several prostanoids namely PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 [53]. TXA_2 is a vasoconstrictor primarily produced by platelets but can also be synthesized in ECs albeit at lower levels [3]. Thromboxane-prostanoid receptors (TP-receptors) are the key players involved in endothelium-dependent contractions since all prostanoids can bind to them but to variable degrees of affinity [54]. Endoperoxides and prostacyclin also activate TP-receptors but lead to transient contractions resembling acetylcholine-induced endothelium-dependent contractions because of their short half-life [55]. TXA_2 is the most potent vasoconstrictor among the prostanoids since it is the preferential ligand for TP receptors activation eliciting several physiological/pathological responses in particular platelet aggregation, expression of adhesion molecules, and smooth muscle contraction [53]. Prostanoids such as TXA_2 and PGH_2 activate G-protein TP receptors on VSMC to cause vasoconstriction by 1) antagonising the action of the vasodilator PGI_2 to lower intracellular cAMP [56]; 2) by activating the downstream Rho kinase pathway increasing VSM contractile activity [55]. In the course of the cascade of reactions that generate prostanoids, COX enzymes also produce ROS including those involved in endothelium-dependent responses such as

superoxide anion ($\cdot O_2$-), hydroxyl radicals·(OH), and hydrogen peroxide (H_2O_2) [55]. ROS can directly act as EDCFs or by indirectly enhancing EDCF-mediated response by inactivating ·NO reducing its bioavailability [57, 58] and stimulating COX located in the VSMC [59, 60]. In addition, reaction of $\cdot O_2$- with ·NO generates peroxynitrite, a strong oxidant that inactivates PGI synthase and therefore shifts the production of prostacyclin to other vasoconstrictor prostanoids [61].

Under normal conditions, the predominant ·NO and EDHF production and PGI_2, the main product of AA metabolism [62], override the effect of the minor amount of vasoconstrictor prostanoids [63]. Nevertheless, the responsiveness to endoperoxides is increased in hypertensive rats (SHR) even though the expression and presence of TP receptors are not altered [64]. Other studies in SHR also show a loss of relaxations in response to prostacyclin despite unchanged expression of IP receptors [65] and a decrease in endothelium-dependent relaxations induced by ACh [66] caused by the release of contracting factors dependent of COX [67].

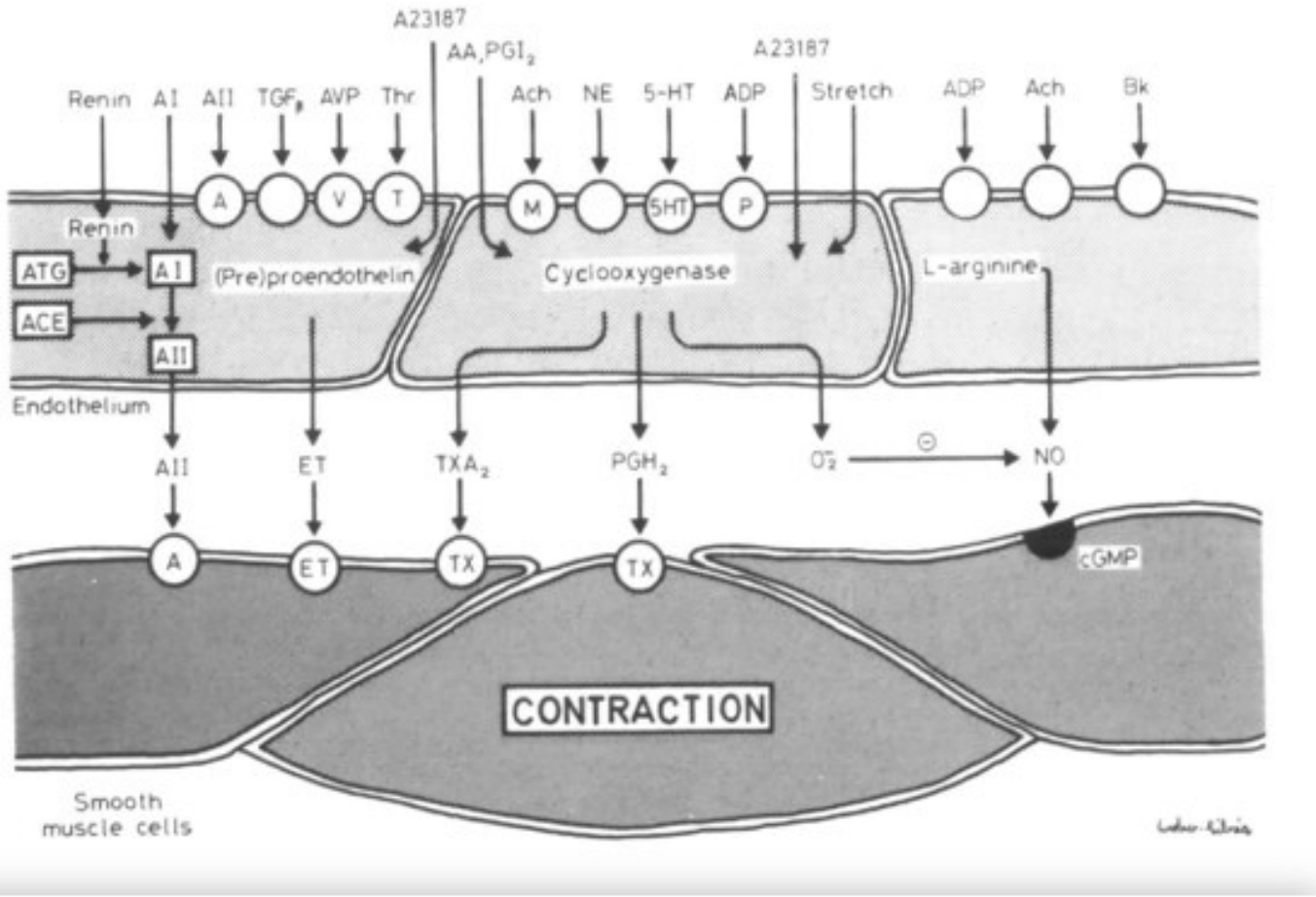

Figure 5. – Various endothelium-derived contracting factors (EDCF) produced in ECs and their action on their smooth muscle receptors to induce vascular smooth muscle contraction.

Cyclooxygenase stimulation by chemical stimuli (Arachidonic acid; AA, prostacyclin, PGI2, acetylcholine; ACh, Norepinephrine; NE, Serotonin, 5-HT, adenosine disphosphate; ADP, calcium ionophore A23187) on their respective receptors, by physical forces, or via the calcium ionophore form the EDCFs thromboxane A_2 (TXA_2), prostaglandin H_2 (PGH_2) or superoxide radical ($\cdot O_2^-$). $\cdot O_2^-$ decreases ·NO production and bioavailability. Angiotensin II (All), epinephrine, and arginine vasopressin (AVP) and coagulation factors such as thrombin (Thr) and transforming growth factor β (TGF_β), and calcium ionophore A23187 all stimulate the production of endothelin (ET) [15, 48]. *Permission to reuse obtained (Annex 1).*

Chapter 2 – Endothelial dysfunction and atherosclerosis

Endothelial dysfunction is known to be the first step in the cascade of events leading to the atherogenesis. Coronary disease is at the source of a plethora of cardiovascular ailments [25] stemming from alterations in endothelial cell functions caused by physical trauma and/or a shift in phenotypes involved in homeostasis and local vascular tone regulation. Clinically endothelial dysfunction is characterized by an impaired endothelium-dependent vasorelaxation in response to endothelium-dependent agonists such as acetylcholine and bradykinine [20]. Increases in ROS are a key event in endothelial dysfunction which decreases synthesis or bioavailability of vasodilators, particularly NO [68, 69] causing production and release of EDCF [25]. The imbalance between vasodilation and vasoconstriction disrupts the endothelial barrier permeability a consequence of EC inflammatory response responsible for hypertension, aging, stroke, disease, obesity, venous thrombosis and intimal hyperplasia [70]. Other behavioral risk factors such as smoking, high cholesterol diet and a sedentary lifestyle also promote the initiation and progression of endothelial dysfunction and development of subsequent cardiovascular diseases.

2.1-Reactive oxygen species (ROS)

Under normal condition, ROS levels remain low and the balance in production and elimination is maintained via antioxidants such as vitamins C and E [71, 72]. Endothelial injury worsens as ROS production becomes uncontrolled and as vasodilator bioavailability decreases [73, 74]. The main sources of ROS include: Uncoupled eNOS, xanthine oxidase, NADPH oxidase and the mitochondrial respiratory chain [75]. The common ROS molecules produced by these sources are anion superoxide ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and radical hydroxyl (·OH). ROS are also responsible for increased endothelial permeability, cell apoptosis and senescence, inflammation, and vascular cell growth and migration [76].

2.1.1-Uncoupled eNOS

When the endothelium is intact, eNOS is the enzyme which catalyzes the reaction that produces the most important EDRF, NO·. Under circumstances such as the depletion of the essential cofactor tetrahydrobiopterin (BH_4), deficiency of the eNOS substrate L-arginine, and/or eNOS S-

glutathionylation, eNOS becomes uncoupled and produces superoxide at the expenses of NO· [77]. Ozaki et al have demonstrated the stoichiometric relationship between endothelial BH_4 and eNOS activity. They increased the expression of eNOS in apolipoprotein E (apoE) knockout animals without simultaneously increasing BH_4 levels, which lead to accelerated atherosclerotic plaque formation [78]. This vicious circle results from low levels of BH_4 and L-arginine or oxidation of BH_4, the uncoupled eNOS transfers electrons to O_2, generating $O_2\cdot^-$ [79]. $O_2\cdot^-$ rapidly reacts with NO· to produce another ROS, peroxynitrite ($ONOO^-$) that oxidizes BH_4 to BH_2 leading to competition with BH_4 for binding of eNOS which causes further stimulation of eNOS uncoupling [75]. S-glutathionylation of eNOS is a reversible protein modification which occurs under both oxidative and nitrosative stress in which the cysteine residues of eNOS are oxidized through thiol-disulfide exchange thus uncoupling eNOS and switching from nitric oxide production to $O_2\cdot^-$ generation primarily at the reductase domain [80].

2.1.2-Xanthine oxidase (XO)

Xanthine Oxidoreductase (XOR) is an enzyme that generate ROS and exists in two forms: Xanthine oxidase (XO) and Xanthine dehydrogenase (XDH) [75]. The difference between the two forms lies in their oxidizing specificity: XDH preferentially transfers electrons to NAD^+ whereas XO directly reduces molecular oxygen producing $O_2\cdot^-$ and H_2O_2 [81]. Xanthine Oxidase is responsible for purine catabolism through the catalysis of oxidation of hypoxanthine and xanthine into xanthine and uric acid, respectively [75, 81].

The XDH form is constitutively expressed in epithelial cells in the liver, intestine and mammary glands while XOR is mainly expressed in endothelial cells [75, 81]. Circulating XOR levels are usually very low but, under pathological conditions, XOR levels rise as they are released from damaged cells and bind to endothelial cells triggering remote organ injury [75, 81]. Under ischemic conditions, XDH is oxidized into XO and ATP is converted into ADP and the XO substrate, hypoxanthine. Upon reperfusion, accumulated hypoxanthine is reduced into $O_2\cdot^-$ and H_2O_2 by XO [82]. However, XO is inhibited by both ·NO and $ONOO^-$ [81]. XOR-derived ROS

may play a part in stimulating NADPH oxidase activity and vice versa, making these two sources of ROS interrelated [75].

2.1.3-NADPH oxidase (NOX)

NOX enzymes comprise six subunits interacting together to form an active enzyme complex that generates important amounts of $O_2^{\cdot -}$ by transferring electrons from NADPH to molecular oxygen [75, 83]. In 1970, Karvoscki et al were the first to discover NOX expression in neutrophils which play an antimicrobial role [84]. Under nonpathological conditions, NOX-released ROS serve as an inflammatory mediator by destroying invading microorganisms in macrophage and neutrophils [75, 85]. The prominence of ROS immunity was highlighted by the discovery of a genetic disorder, chronic granulomatous disease (CGD), in which patients have a defect in NOX2 or associated subunits [86]. The patients are not only hypersensitive to bacterial and fungal infections but also have diminished bactericidal capacities and cells antimicrobial action due the inherited inability to produce ROS. The ladder results in the accumulation of bacteria-containing phagocytes leading to the formation of granulomas [87, 88]. Other NOX isoforms were later identified in non-phagocytic cells including ECs, SMCs, as well as in the adventitia [75, 89]. Baseline amounts of ROS generated by NOX are crucial to regulate blood pressure for maintenance of cardiovascular homeostasis, however, the slightest deviation from normal ROS levels are detrimental [90, 91]. NOX enzymatic activity in EC, SMCs, and adventia increases upon Ang II binding [75, 89]. Thus, overstimulation of the renin-angiotensin system, a common characteristic of endothelial dysfunction and hypertension, leads to a state of increased oxidative stress.

2.1.4-Mitochondrial respiratory chain

Mitochondrial oxidative phosphorylation is the metabolic pathway by which electrons are transferred through an electron transport chain (ETC) to generate ATP and O_2. This process is also the main source of cellular ROS, even under physiological conditions, since a small portion of electrons leaks out of the ETC to interact with molecular oxygen to yield $O_2^{\cdot -}$ and H_2O_2 [92]. As mentioned previously, a basal amount of ROS is important as ROS act as second messengers and mediate crucial cellular transformations such as apoptosis, autophagy, necrosis and pyroptosis [92]. However, high levels of ROS are harmful and cause deleterious processes including DNA damage, cardiomyocyte death leading to heart failure, stimulation of sodium-hydrogen exchanger1 (NHE-1) implicated in cardiac hypertrophy, activation of the NF-κB pathway responsible for

inflammation and atherogenesis, and ROS bursts during reperfusion responsible for ischemia-reperfusion injury [93-95]. Crosstalk occurs among ROS sources in the vascular space, especially between NOX and mitochondria [96]. Conversely, Ang II increases ROS generation in mitochondria in a NOX-dependent manner and vice versa [97].

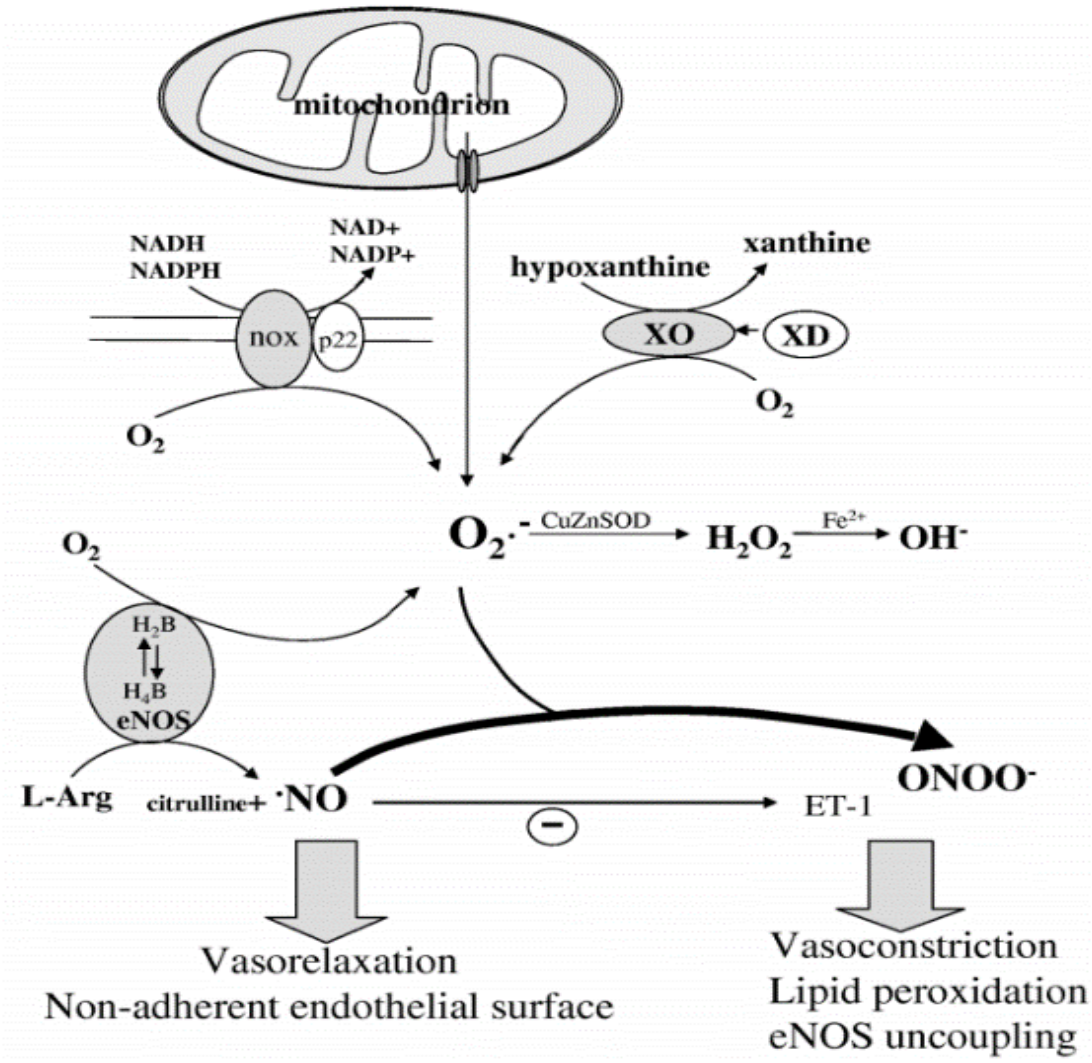

Figure 6. – ROS production under pathological conditions.

Under normal conditions, eNOS produces the potent vasodilator ·NO and the by-product citrulline from the substrate L-arginine. Under pathological conditions such as endothelial injury, ·NO and $O\cdot^{-2}$ drives ROS production via uncoupled eNOS, xanthine oxidase, NADPH oxidase, and the mitochondrial respiratory chain. The production of peroxinitrite ($ONOO^-$) decreases NO bioavailability and endothelium-dependent vasorelaxation, increases lipid peroxidation, and eNOS uncoupling. In the presence of metal ions, $O_2\cdot^-$ can be converted to H_2O_2 which is further metabolized to the hydroxyl radical OH. L-Arg, L-arginine; BH_2, dihydrobiopterin; BH_4, tetrahydrobiopterin; XD, xanthine dehydrogenase; SOD, superoxide dismutase[71]. *Open access article - no permission required (Annex 1).*

2.2-Atherosclerosis

Atherosclerosis is a chronic arterial disease in which an arterial plaque develops consisting of lipids, cholesterol, calcium, and other substances found in the blood. These plaques occur in the blood vessel walls causing luminal narrowing and eventually reducing blood flow. Atherosclerosis is multifocal in distribution and early lesions usually originate in areas with low or oscillatory shear stress such as bifurcations sites, branching points, convex areas of bending arteries [98, 99]. Under normal vascular physiology, ·NO exerts highly critical cardioprotective functions by maintaining the vascular wall in a quiescent state and impeding inflammation, cellular proliferation, and thrombosis. However, predisposing risk factors such as hypertension, diabetes, smoking, and hypercholesterolemia are associated with functional impairment of the endothelium [100]. Increases in blood low-density lipoprotein (LDL) such as in hypercholesterolemia or other genetic dyslipidemias are common causes of atherosclerosis. Most often atherogenesis is a multifactorial disease as it may develop at low levels of LDL in the presence of other risk factors [101]. Prolonged exposure to cardiovascular risk factors lead to physical trauma or more subtle cellular damage known as endothelial injury or endothelial dysfunction, which constitutes the initial event in atherogenesis [2, 4]. Lüdmer et al. provided evidence of the presence of endothelial dysfunction in the early stage of atherosclerosis where they observed impaired endothelium-dependent vasodilation in response to acetylcholine in atherosclerotic coronary arteries [102, 103]. In fact, paradoxical constriction occurred in patients with both mild and advanced coronary diseases indicating endothelial dysfunction occurrence in early stage of atherosclerosis [102]. Furthermore, acetylcholine tests or FMD measurements corroborated the presence of endothelial dysfunction at the conduit and microvascular level in patients with coronary disease risk factors even though imaging of coronary vasculature (angiography and ultrasound) showed no structural evidence of structural CAD [104, 105].

2.2.1- Endothelial activation & fatty streak

Endothelial dysfunction is also known as endothelial activation, a state during which a switch occurs from a quiescent phenotype (normal NO generation) towards an immunological response resulting from cardiovascular risk factors initiating expression and production of inflammatory molecules such as chemokines, cytokines, and adhesion molecules [4]. eNOS that initially produced NO to maintain endothelial quiescence, becomes uncoupled and shifts to

producing ROS [4]. Excess ROS production resulting from endothelial function impairment leads to an attack of cellular membranes and organelles membranes which have a high content in high polyunsaturated fatty acids (PUFAs) [106]. Lipid peroxidation is a reaction by which free radicals remove electrons from lipids producing highly reactive intermediates that undergo further reactions to produce lipid hydroperoxides (LOOH), malondialdehyde (MDA), propanal, hexanal, and 4-hydroxunonenal (4-HNE) [107]. Peroxidised lipids can directly act as cell death signal inducing programmed cell death (apoptosis) or mediate proinflammatory changes in several inflammatory diseases [106, 108]. The prominent impairment of endothelium-dependent relaxation in human coronary arteries is manifested by G_i-protein dysfunction, due to its decrease expression [109]. Decreases in NO synthesis and eNOS expression [110], and increases in ET-1 production, as reflected by the raise in ET-1 mRNA levels, are three alterations sparked by peroxidised lipids [111]. In advanced stages of atherosclerosis, eNOS expression is reduced due the presence of the cytokine TNF-α in atherosclerotic plaques which shortens the half-life of eNOS mRNA [112]. In addition, oxidized LDLs specifically diminish L-arginine bioavailability in the coronary microcirculation of hypercholesteraemic patients since endothelial vasodilator response to ACh are maintained with NO donor agonists and partially restored upon L-arginine supplementation [112].

Endothelial activation causes phenotypic alterations that render the endothelium more permeable to solutes and prone to leukocyte and monocyte adhesion due to the increase in expression of leukocytes adhesion molecules (VCAM-1) and the cytokine monocyte chemoattractant protein-1 (MCP-1) [3]. Leukocytes, monocytes, platelets and lipoproteins accumulation in the vessel walls paired with smooth muscle cell migration and proliferation promote the development of fatty streaks [113-115]. Fatty streaks are the first lesions to be visible to the naked eye since they appear as yellow deposits inside the vessel wall and consist of cholesterol and white blood cells.

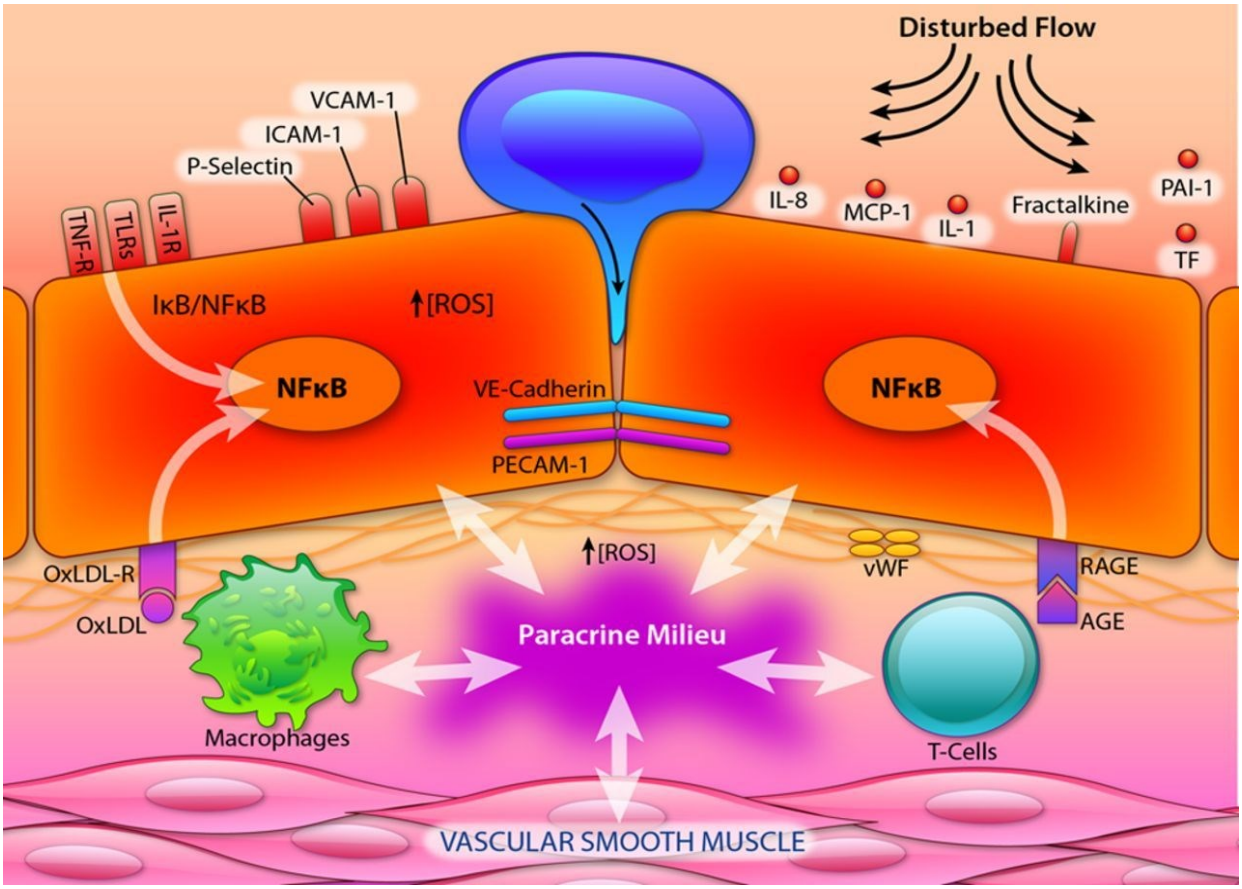

Figure 7. – Endothelial activation

Proinflammatory agonists and conditions including IL-1, TNF, endotoxins, oxidized lipoproteins (ox-LDL), advanced glycation end products (AGE), disturbed blood flow, elicit endothelial activation via the pleiotropic transcription factor nuclear factor-κB (NF-κB). NF-κB increases the expression of various effector proteins responsible of genetic regulation within the endothelial cells as well as cell surface expression of adhesion molecules (VCAM-1), secreted and membrane-associated chemokines (MCP]-1 and fractalkine), and prothrombotic mediators (tissue factor [TF], vonWillebrand Factor [vWF], and plasminogen activator inhibitor [PAI]-1). These events promote the recruitment of monocytes and T lymphocytes, which enter the subendothelial space via diapedesis. A Chronic proinflammatory state is maintained via the production of a complex paracrine milieu of cytokines, growth factors and ROS within the vessel wall and leads to progression of atherosclerotic lesions. IL-R, TNF-R are receptors for IL-1, TNF, respectively; Ox-LDL-R, receptors for oxidized LDL; RAGE, receptors for AGE; and TLRs are Toll-like receptors[116]. *Permission to reuse obtained (Annex 1)*

2.2.2-Type I and Type II endothelial activation

Type I endothelial activation is a rapid and self-limited condition and refers to the action of histamine and other phlogistic agents eliciting the cardinal signs of inflammation (rubor, calor, tumor, and dolor) [117].

Type II endothelial activation results from the expression of pleiotropic transcription factors such as the nuclear factor-κB (NF-κB), a common denominator in vascular disease, that results in the expression of various effector proteins [116]. Monocytes recruitment occurs early in formation of atherosclerotic plaque due to the presence of chemoattractants. Leukocytes recruitment occurs later as they only adhere to an activated endothelium [3]. Following rolling and adhesion on the activated endothelial surface, monocytes undergo diapedesis and differentiation and begin internalizing oxidized lipids in the intimal vessel wall becoming foam cells by accumulating lipid droplets [3]. The fatty streaks continues expanding causing macrophage apoptosis, matrix accumulation and plaque growth [3].

2.2.3-Stable (fibrous) or unstable plaque formation and lesion rupture

SMCs secrete a matrix-rich in collagen, elastin and proteoglycans that separates the liquid core of the atherosclerosis plaque from circulating blood known as the fibroatheromatous plaque [3]. Current understanding suggests that fibrous caps is an evolved version of fibroatheromatous cap. The thickness and morphology of the fibrous cap determines its stability. Cap thickness is usually determined by the ratio of biosynthesis vs degradation of collagen, the main component, by a family of matrix-degrading metalloproteinase (MMP) enzymes [118, 119]. In fact, caps containing less matrix elements and SMC are thinner, more unstable, and have a propensity to rupture causing the top two cardiovascular killers events worldwide: myocardial infarction and stroke [120].

2.3-Clinical assessment of endothelial function

Arterial endothelial dysfunction precedes structural atherosclerotic changes that slowly progress to eventually cause luminal stenosis and clinical symptoms. Invasive and non-invasive methods have been developed to clinically assess endothelial function. The gold standard test for assessing coronary endothelial function is the invasive intracoronary acetylcholine (IC-ACh)

provocation testing [121]. The first step of this two-step test is administration of adenosine into one of the coronary arteries to achieve small vessel dilation and for measurement of blood flow [122]. Secondly, acetylcholine is injected to induce both endothelial-dependent dilation along with smooth muscle-mediated constriction. In healthy individual, endothelium-dependent dilation predominates whereas in the presence of endothelial damage vasoconstriction prevails. Additionally, coronary angiography, an X-ray imaging test is used before and after ACh injection to quantitively compare coronary artery diameters [2].

Unfortunately, invasive methods are unsuitable to use in young asymptomatic subjects, which called for the development of non-invasive methods:

High-resolution ultrasound is a non-invasive method to assess endothelial function and measures changes in the diameters of superficial arteries in response to reactive hyperemia [123]. Reactive hyperemia results in elevated shear stress as a consequence of increased blood flow, which stimulates the production of endothelium-derived vasodilators. Ultrasound imaging is used to quantify the changes in arterial diameter caused by flow-mediated dilation (FMD) following shear stress and estimate endothelium-derived vasodilator release [115].

Measurement of C-reactive protein (CRP) is another non-invasive method as CRP levels inversely correlate the adequacy of the endothelial function [124]. However, elevated CRP levels indicate systemic inflammatory responses that might be associated with other conditions than coronary endothelial function.

Venous occlusion plethysmography is an invasive method that measures changes in blood flow in forearm vessels upon ACh infusion in the brachial artery [123]. Atherosclerosis is a clearly systemic disease since there is close correlation between endothelial dysfunction in the forearm and coronary endothelial dysfunction [103, 123].

2.4- Endothelial dysfunction reversal and pharmacological treatment

Luckily, endothelial dysfunction in the early stages appears to be a reversible process which can respond to changes in lifestyle and to various pharmacological agents [116]. For instance, 75%

of sudden cardiac deaths due to acute thrombosis are observed in cigarette smokers [125]. In healthy young adults, passive smoking is associated with dose-related impairment of endothelium-dependent dilation indicating early arterial damage [126]. Moreover, a healthy diet combined with exercising contribute to the maintaince of a healthy weight and decrease the risk of developing diseases such as type 2 diabetes and hypertension. Thus, smoking cessation and addressing lifestyle risk factors are priorities in order to help patients with or at risk of contracting various cardiovascular diseases.

Current pharmacological intervention consists of different drugs with various targets and focus mainly on improving known risk factors for cardiovascular disease rather than targeting specific endothelial-based mechanisms [20, 116].

Statins are a class of drugs used to reduce blood cholesterol by inhibiting the enzyme hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase. Moreover, statins not only increase NO bioavailability by stimulating eNOS via PI3K/Akt signaling pathway [127], they also have antioxidant effects by reducing the electronegative form of LDL [128, 129].

Angiotensin converting enzymes (ACE) inhibitors and **angiotensin receptor blockers (ARBs)** are used to treat hypertension and heart failure by decreasing the levels and effects of angiotensin II and increasing bradykinin levels [20]. They also exert endothelial protective effects via inhibition of ROS and COX-2 derived vasoconstrictors production [130].

Beta blockers (BB) were initially introduced in 1960s as antihypertensive agents and as a treatment against coronary artery disease and heart failure [131]. The BB Nebivolol (β1 antagonist with β2,3-agonist property) improves endothelial function and when it was administered to smokers and patients with essential hypertension, improved endothelium-dependent vasodilation responses were observed [20]. A meta-analysis concluded that BB significantly improved endothelial function compared with placebo, but this effect was indirect as there is no evidence that BB or their metabolites specifically interact with endothelial cells [131].

Dihydropyridine calcium channel blockers also protect the endothelium primarily via their antioxidant actions against ROS associated with endothelial cell death and by reducing lipid

peroxidation [132, 133]. Amlodipine combined to a renin inhibitor improves the endothelial function of patients with hypertension, mainly due to its NO-releasing actions and anti-inflammatory effects [134, 135]. Bradykinin has a short-life and plays a role in inflammation and cancer making its use as a treatment for endothelial dysfunction challenging [136, 137]. Conversely, bradykinin preserves eNOS expression and delays the progression of heart failure in dogs [138] in addition to protecting from ROS and toxin-induced microvascular endothelial cell death [139].

Antioxidants such as vitamin C and vitamin E can reverse endothelial dysfunction as they scavenge superoxide, thus preventing NO scavenging, lipid peroxidation and all subsequent adverse effects. In patients with chronic smoking, diabetes mellitus hypercholesterolemia and hypertension, vitamin C improves endothelium-dependent vasodilation [140-142]. Finally, several other pharmacological agents have been proposed to treat endothelial dysfunction such as the novel therapies including ranolazine, aminophylline, and other experimental therapies, but the use of such drugs depend on the risk factors causing the condition.

2.5- Surgical treatment of coronary artery disease

Atherosclerosis affects all arteries in the body. Coronary microvascular disease (CMD) occurs when plaques form in the coronary microcirculation whereas coronary artery disease (CAD) usually refers to plaque build up in the main epicardial coronary arteries. In advanced coronary artery disease, the myocardial blood flow is reduced making patients predisposed to develop blood clots that can partially or completely block blood flow causing chest pain known as angina or ultimately myocardial infarction. Percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) surgery are the main treatments for CAD to restore myocardial reperfusion in patients that are unresponsive to vasodilatory drugs such as nitroglycerin. Both revascularization procedures are always combined to pharmacological therapy to reduce cardiac workload, promote vasodilation, and treat symptoms such as chest pain (angina) and shortness of breath. The SYNTAX randomized trial (Synergy between PCI with Taxus and Cardiac Surgery) compared 1st generation drug-eluting stent PCI (paclitaxel-eluting stent) vs CABG, in 1800 patients with de novo three-vessels left main coronary artery disease. Subsequent studies, SYNTAXES (SYNTAX Extended Survival) reported 5 and 10-year follow-up of these patients [143, 144]. This

trial is the landmark in decision making between PCI and CABG as it introduced the SYNTAX score as means of grading patient's coronary disease progression [145]. All these studies have reported that CABG patients experienced less cardiac and cerebrovascular events, in addition to a survival advantage when compared to PCI patients. However, contemporary guidelines to use the appropriate revascularisation techniques and improved drug therapy such as the dual platelet therapy have shown improved results after PCI in extensive CAD [146].

2.5.1- Percutaneous coronary intervention (PCI)

Percutaneous coronary intervention is an invasive procedure in which a catheter is inserted into the stenosed coronary artery to deploy a stent, which will stabilize and compress the plaque to widen the narrowed or blocked coronary arteries [147]. Traditionally, balloon angioplasty was a technique in which a small balloon is inflated at the site of coronary occlusion to reinstate blood flow [147, 148]. However, early studies reported a high death rate when compared to CABG due to endothelial damage leading to arterial wall weakening and restenosis [149] prompting a higher occurrence of major adverse events such as myocardial infarction and stroke [150]. Bare-metal stents (BMS), drug-eluting stents (DES), and bioresorbable stents (BRS) are types of stents developed to amend the shortcomings of the traditional method even though each one has its own flaws, but the choice of the device remains dependent on the lesional and anatomical requisites.

2.5.2- Coronary artery bypass grafting (CABG) surgery

CABG procedure is the standard of care for symptomatic patients with coronary disease since its introduction in 1968 [151]. Several indications call for CABG surgery such as three-vessel disease; left main disease with associated distal vessel disease, and isolated left main disease involving distal segment or bifurcation and coronary ostial stenosis [152]. Pathological conditions less suitable for PCI including total occlusion, circumference calcification, and obstructive CAD associated with cardiac dysfunction also requires CABG surgery [152, 153]. The surgeon uses autologous harvesting of coronary conduits namely the internal thoracic artery (ITA), the greater saphenous vein, radial artery, gastroepiploic artery for revascularisation by rerouting the coronary blood flow and bypassing the coronary lesions [152]. Saphenous veins remain one of the most widely used conduit since it is readily available and easy to harvest even though it has reported lower long term patency rates than arterial grafts, particularly the left ITA. [154, 155].

To conduct this procedure, the patient is put under general anesthesia. Among other techniques, saphenous vein grafts (SVG) are usually harvested using state-of-the-art techniques (open or endoscopic or no touch) and handling techniques of (i.e. atraumatic surgical technique, avoidance of over pressurization during checking for leakage, excessive handling and distortion) to achieve minimal traumatic damage to the conduit's endothelium [156]. The segment is carefully flushed and stored within a preservation solution. Meanwhile, sternotomy is performed to allow the surgeon to access the heart. During CABG, drugs are administered to stop the heart (cardioplegia) and allow the surgeon to easily connect the unobstructed vessel grafts and bypass the blocked portions of the coronary artery. Once grafting is completed, blood flow is restored and the heart usually restarts beating by its own or sometimes using mild electric shocks defibrillation or cardioversion[157].

2.6- Venous graft failure

Saphenous vein grafts are the most used conduits in coronary artery bypass grafting (CABG) even with a suboptimal one-year primary patency of 61% as reported in the PREVENT III trial [158, 159]. Injury to SVGs during harvesting and storage promotes endothelial dysfunction, which is attributed to a decrease in production of nitric oxide and/or increased level of oxidative stress that can progress to vein graft failure (VGF). The low long-term patency rate of autologous human saphenous vein grafts use in CABG surgery is mainly due to intimal hyperplasia (IH) [160, 161] and atherosclerosis [162] leading to vessel lumen narrowing, graft stenosis and occlusion [163]. These pathological processes ultimately cause venous graft failure (VGF) both in the short term (30 days to 2 years) and long term (> 2 years), respectively [162]. Venous wall adjustment to a greater intraluminal pressure of arterial blood flow and endothelial trauma incurred to SV conduits during back-table graft preparation, namely storage in acidic or non-buffered preservation solutions, usage of surgical skin markers and pressure distension when flushing the vein with uncontrolled pressure by handheld syringes, are among the foremost contributors to endothelial denudation that potentiate inflammatory responses leading to IH [161, 162, 164-169]. Recently, many studies found that the preservation solutions used until anastomosis plays a major role in VGF occurrence.

Chapter 3 – Graft preservation solutions

Each year, approximately 1 million aortocoronary and peripheral vascular operations are performed using human saphenous vein (HSV) [162, 170]. High rates of graft failure are primarily caused by intimal hyperplasia (IH) limiting the durability of the venous conduits and significant morbidity, reintervention, limb loss, myocardial infarction (MI) and death (Osgood 2014). Successive and additive vein graft injury are generated by the following surgical manipulations : harvesting, mechanical stretching, spotting leaks by vessel pressure distention via hand-held syringe, labelling conduit orientation using toxic surgical skin markers (isopropyl alcohol solvent, a component in the ink) [164], and storing in an acidic solution. Most studies address endothelial damage caused by harvesting techniques and found that early graft failure that happens within 30 days is mostly due to those surgical techniques [171-173]. Beyond the time frame of a month and up until a decade, vascular graft disease causes graft failure [174] manifested as intimal hyperplasia and atherosclerosis [175].

Several studies have investigated the effects of different intra-operative short time graft storage solutions to determine if components such as the pH, temperature, duration of storage, and composition of these solutions affect graft patency and have allowed to estimate optimal storage conditions. Upon implantation, venous graft goes through a series of adaptation to the new higher blood flow via early lumen dilation followed by vessel wall thickening and stiffening [169]. The maintenance of an intact endothelial layer is crucial, as it is a prerequisite to maintain adaptive vascular reactivity upon the secretion of physiologically vasoactive substances. Thus, the widespread of CABG procedure warrants a close overview of every individual component that threatens a positive clinical outcome such as the detrimental effects caused by preservation solutions to SVG. The choice of intraoperative storage solution significantly affects endothelial structure safeguarding and vascular function [176-191]. There is major lack of clinical studies investigating the effects of preservation solution on SVG patency illustrated by the lack of consensus and variation in the use of vein graft storage solutions in different cardiovascular surgery centers. Evidently, it is less complicated and more cost effective to create optimal conditions to

promote graft patency upon coronary bypass grafting, then to reoperate attempt treatment of an occluded graft [192].

3.1- Optimal tissue storage and confounding conditions

According to the association of Organ Procurement Organization, the main goal of preservation should be the mitigation of ischemic damage during the storage period by hindering either oxidative damage or metabolic stress [192]. The Prevent IV randomized clinical trial, is a major study that evaluated the contribution of preservation solutions to VGF and the clinical outcomes in CABG patients [169]. Harskamp and colleagues investigated the preservation of vein grafts in saline, blood or buffered saline solutions by performing a one-year angiography to evaluate VGF in addition to reporting 5-year rates of death, myocardial infarctions, and subsequent revascularisations. The standard of care (SOC) preservation solution, saline, had both the highest percentage of patient-level VGF (46.5%) and graft-level VGF (27.0%) whereas the buffered saline solution showed a greater vein graft patency and trends towards improved clinical outcomes over the 5-year follow-up.

3.1.1- pH

Biswas et al determined both the temporal and pH effects on vein segment endothelial and smooth muscle cell viability via the measurement of bradykinin activation of eNOS, with or without N-nitro-L-arginine, using multiphoton imaging [193]. They observed that cell viability was preserved up to 3h of storage time but substantially decreased after 4h of storage. Moreover, cell viability was maintained at physiological pH (7.4) but was dramatically reduced at pH 8.0 and completely lost at pH 6.0.

3.1.2- Temperature

Storage solution temperature is another factor that has an impact on endothelial activation, which can trigger chronic inflammation and subsequent obliterative lesions followed by graft occlusion [194]. Preserving allografts at 4ºC results in exfoliation of endothelial cells and decrease the amount of smooth muscle cells in the medium [195]. Wille et al reported porcine aortae cold-

induced iron-dependent injury that are further enhanced during rewarming [196] and proposed iron chelators and optimized base solutions to improve vascular tissue storage.

3.1.3- Storage solutions biocompatibility

The composition of preservation solutions is crucial to the maintenance of endothelial and tissue integrity. For instance, endothelial cells are vulnerable to oxidative damage, thus, an optimal preservation should contain antioxidants to diminish oxidation from the ROS released by cells including endothelial cells [71, 192]. Biocompatibility of the storage solution to the tissue to which it was designed to preserve is also important in order to avoid adding 'solution damage' to the ischemic damage previously inflicted [192]. However, general confounding factors such as temperature and storage time need further ex-vivo investigation of their effect on graft endothelial preservation.

3.2- Heparinized 0.9% normal saline solution (NS)

Saline solution is still widely used even though numerous studies have reported deleterious effects on endothelial function. It is a hypertonic and acidic solution (pH 5) that contains 40 U/mL of heparin, 150 mmol/L of sodium chloride. NS only contains sodium and chloride, and does not contain any endothelial protective component. Detrimental effects of normal saline were recognized as early as 1974 when O'Connell et al demonstrated that preserving rabbit carotid arteries for 2-3 hours in saline infusion produced increased myointimal proliferation and obliteration 1 month post operatively relative to serum-infused control [197]. The Gi-mediated pathway is among the first endothelial pathways to be impacted in disease and is known to be an early marker of pathological endothelial activation involved in vascular diseases [198]. Upon perfusion and preservation of porcine coronary arteries in saline, Perrault et al hypothesized that saline solution does not allow for recovery of the Gi-protein-mediated relaxations as shown by the decrease in responses to serotonin [198].

In 2013, Wilbring et al clearly recommended to stop using NS as storage solution and use other solution designed to reduce loss of endothelium-dependent vascular function during cold-

storage [190]. Several studies have compared the effects of storage in NS vs other solutions and have reported vein graft damage and dysfunction [199]. NS does not contain a source for cellular energy [184]. It also promotes leukocytes and thrombocyte adhesion with consecutive inflammatory reactions which plays a central role in the development of graft atherosclerosis [200]. Among other things, NS does not directly intervene to attenuate oxidative damage and metabolic stress mediating ischemic injury [192]. NS also inflicts 'solution damage' in addition to ischemic damage since it is to not be biocompatible with the tissue [185, 189, 192]. Moreover, heparin added to normal saline is toxic to the graft and was shown that it can induce thrombocytopenia even at low doses as well as other coagulopathies [201, 202]. Due to its acidic pH, the absence of endothelial protective additives, its lack of biocompatibility, and the studies that clearly report its negative effects on SVG, NS should not be used as a storage solution. Other than the pH, the mechanism behind the harmful effects in NS might include its mild hyperosmolarity, lack of cellular nutrients and antioxidants [197].

3.3- DuraGraft®/GALA solution

GALA is a preservation saphenous graft preservation that was first developed and used at the Veterans Affairs (VA) laboratories in Boston by Dr Thatte and Dr Khuri in early 2000s. The acronym GALA stands for its components: Glutathione, ascorbic acid, L-arginine. The GALA solution had major instability issues as glutathione and ascorbic acid degraded in under 36 hours, so it has to be mixed close to time of usage. The solution GALA became the precursor for the commercialized product DuraGraft®. GALA is now made of two solutions: solution A contains salts and solution B has oxygen control components. Solution A has no stability issues and is made of potassium, magnesium, calcium, chloride, bicarbonate, phosphate, and sulfate. Solution B contains all the instable components such as glutathione, ascorbic acid, L-arginine, and glucose. Somahlution has implemented several drug manufacturing control steps such as contamination control, bioburden measurement and oxygen control. For instance, solution B undergoes oxygen replacement by argon gas to prevent antioxidant oxidation and degradation. Another challenge that was faced was finding a system to obtain physiological pH upon mixing of solution A and B mixing. Solution B containing oxygen-controlled substances has a lower pH, but upon mixture

DuraGraft® has the same intended use and composition as the precursor solution GALA. DuraGraft® is currently approved in Europe and is still under FDA review at the present time.

3.3.1- Composition of DuraGraft®

Unlike other preservation solutions, every component of DuraGraft® was specifically added for the sole purpose of protecting the endothelial layer lining the lumen of the graft. The precursor GALA solution was based on Hank's balanced saline solution [200]. Reduced glutathione, L-ascorbic acid, and L-arginine have putative effects on endothelial cell function and act synergistically to enhance endothelial layer protection GALA [184]. L-arginine is the substrate of eNOS, and thus providing greater quantities helps stimulate eNOS activity in addition to decreasing neutrophil-endothelial cell interactions in inflamed vessels [184, 203]. L-ascorbic acid (Vitamin C) is an antioxidant that scavenges ROS which increases NO availability and helps maintain long term NO release to enhance vessel patency during storage in GALA [184]. Reversal of endothelial dysfunction is mediated by L-ascorbic acid via reduced platelet activation, leukocytes adhesion, inhibition of smooth muscle cell proliferation and lipid peroxidation [204, 205]. Glutathione is another powerful antioxidant and reducing agent that helps transport L-arginine into endothelial cells to stimulate eNOS activity, and therefore increasing NO bioavailability and coronary vasodilation [203, 206]. The salts and electrolytes found in solution A mimic the composition of physiological blood, while glucose acts a cellular energy fuel. Other than GALA, table 1 summaries the composition of other graft solutions that are also commonly used.

Solutions	pH	Electrolytes	Additives
Standard solution			
0.9% normal saline	4.5 – 7.0	Na^+: 154 mmol/L Cl^-: 154 mmol/L	None
Autologous heparinized blood (AHB)	7.4	Na^+: 140 mmol/L Cl^-: 110 mmol/L K^+: 5 mmol/L Mg^{2+}: 2 mmol/L SO_4^{2-}: 2 mmol/L	None
Buffered solution			
GALA	7.4	Na^+: 142-156 mmol/L Cl^-: 145 mmol/L K^+: 5.8-6 mmol/L Mg^{2+}: 0.89-1 mmol/L Ca^{2+}: 0.95-1 mmol/L SO_4^{2-}: 0.5 mmol/L HCO^{-3}: 4 mmol/L	Glutathione, L-ascorbic acid, L-arginine, glucose
TiProtec™	7.0 at 20°C	Na^+: 16 mmol/L Cl^-: 103.1 mmol/L K^+: 93 mmol/L Mg^{2+}: 8 mmol/L Ca^{2+}: 0.05 mmol/L	α-ketoglutarate, aspartate, N-acetylhistidine, glycine, alanine, tryptophan, sucrose, glucose, deferoxamine, 3,4-dimethoxy-N-methylbenzohydroxamic acid
University of Wisconsin (UW)	7.4	Na^+: 27 mmol/L K^+: 105 mmol/L Mg^{2+}: 5 mmol/L SO_4^{2-}: 5 mmol/L	Lactobionic acid, adenosine, allopurinol, raffinose, glutathione, polyhydroxyethyl starch
He Solution	7.4	Na^+: 147 mmol/L Cl^-: 156 mmol/L K^+: 4 mmol/L Ca^{2+}: 2.3 mmol/L HCO^{-3}: 0.2 ml of 8.4%	Verapamil, hydrochloride,glyceride trinitrite, heparin
Histidine-tryptophan-Ketoglutarate (HTK)	7.0 at 4°C	Na^+: 15 mmol/L Cl^-: 50 mmol/L K^+: 9 mmol/L Mg^{2+}: 4 mmol/L Ca^{2+}: 0.015 mmol/L	Histidine, α-ketoglutarate, tryptophane, mannitol
Plasmalyte A (Baxter)	7.4	Na^+: 140 mmol/L Cl^-: 98 mmol/L K^+: 5 mmol/L Mg^{2+}: 3 mmol/L $[C_2H_3O_2]^-$: 27 mmol/L $C_6H_{12}O_7$: 23 mmol/L	None

Tableau 1. – Other solutions used for SVGs storage during CABG surgery. Adapted from Ben Ali et al.[199]

3.3.2- Fundamental studies investigating the effects of GALA on vessel patency

Currently, only three research studies have investigated the effects on GALA on endothelial functions and structure in CABG grafts. Thatte and colleagues published an article in 2003 comparing the effects of multiple preservation solutions on cellular viability, intracellular calcium mobilization, nitric oxide generation, and endothelium structural integrity in human saphenous veins. The solutions studied were heparinized lidocaine saline (HLS), autologous heparinized blood (AHB), tissue culture medium (TCM), Hank's balanced salt solution (HBSS), and GALA. Figure 8 exhibits changes in SVG structural integrity with each of the one the preservation solution through time. The segments stored in GALA kept an intact structure and the cells remained viable as illustrated by green fluorescence even after being stored in GALA for 24 hours. Vein segments preserved in the other solutions showed vessel structural disintegration and cellular death marked with red fluorescence after 1-hour storage. Figure 9 shows transverse sections of SV viewed with multiphoton microscopy in transmission mode after storage in HLS, AHB or GALA for 60 minutes. The structure of the saphenous vein preserved in GALA remained intact in contrast to the other two solutions. Furthermore, vein segments were stored in the five preservation solutions at different time points incubated with diaminofluorescein (DAF) to measure NO generation. GALA preserved eNOS activation and NO generation while being severely impaired in all other solutions (figure 10).

Patchuk and colleagues recently published an article in 2019 where they evaluated cell viability and morphology in human saphenous vein (HSV) submerged in saline versus GALA. They also performed histological assessments of isolated porcine mammary veins (PMVs) stored in saline versus GALA. PMVs were chosen as an ex-vivo model because they are similar to HSVs in their size and architecture [192]. In figure 11 below, multiphoton micrographs showed cell death in HSVs with post-exposure to saline for 15 min (c) whereas cell viability was maintained in HSVs submerged in GALA for 1 hour (a) [192]. Histological analysis of PMVs stored in saline for 24 hours displayed multifocal aggregation of endothelial cells, missing patches of the endothelial layer, and weak CD31 and Von Willebrand Factor (vWF) staining. PMVs submerged in GALA for 24 hours showed continuous immunostaining of across the endothelial layer with strong staining

of the two endothelial cell surface markers CD31 and vWF attesting to endothelial cells preservation.

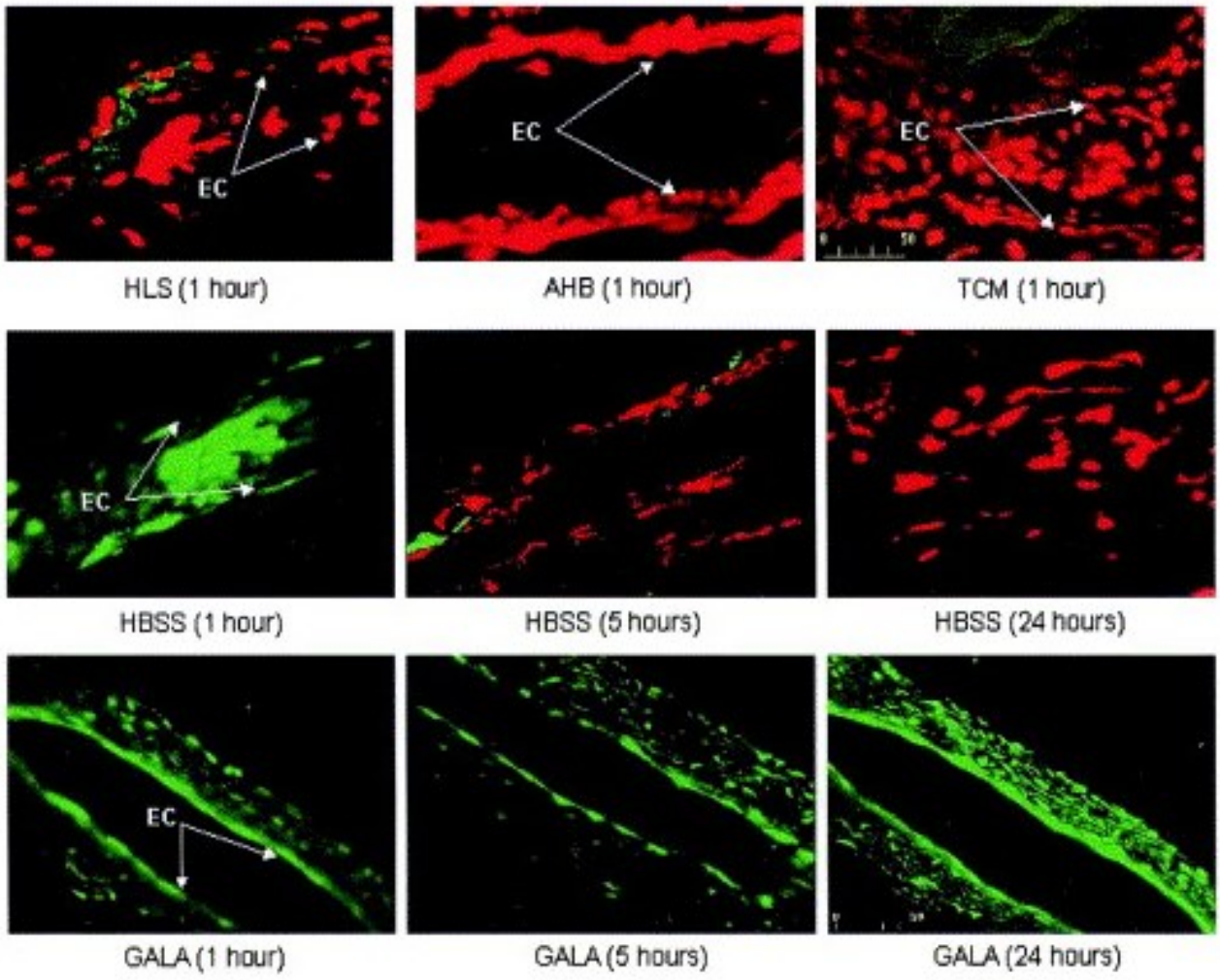

Figure 8. – Structural integrity and cellular viability evaluation of Human saphenous vein segments after storage in various preservation solutions.

The red fluorescence represents non-viable cells while green fluorescence illustrates live cells. Extensive endothelial cellular death and compromised structural integrity were observed in heparinized lidocaine saline (HLS), autologous heparinized blood (AHB), tissue culture medium (TCM) after 1 hour of storage and in Hank's balanced salt solution (HBSS) after 5 hours of storage. Even after 24 hours of storage in GALA, vein graft structural integrity and cellular viability were maintained [184]. *Permission to reuse obtained (Annex 1).*

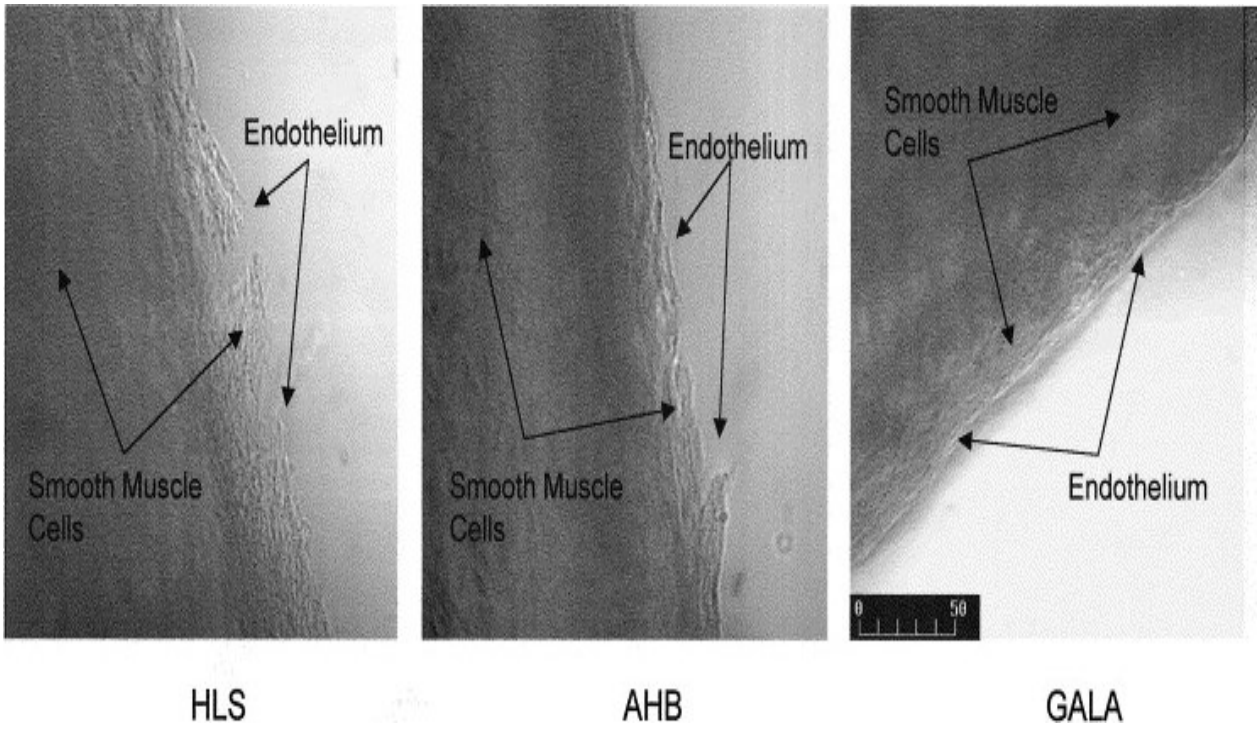

Figure 9. – Multiphoton microscopy in transmission mode of transverse sections of human saphenous vein.

SVGs stored in heparinized lidocaine saline (HLS), autologous heparinized blood (AHB), and GALA for 60 min. Disruption of the endothelial layer and smooth muscle cell layer damage were observed with storage in HLS and AHB. Saphenous segments stored in GALA showed good preservation of endothelium and smooth muscle cell integrity [184]. *Permission to reuse obtained (Annex 1).*

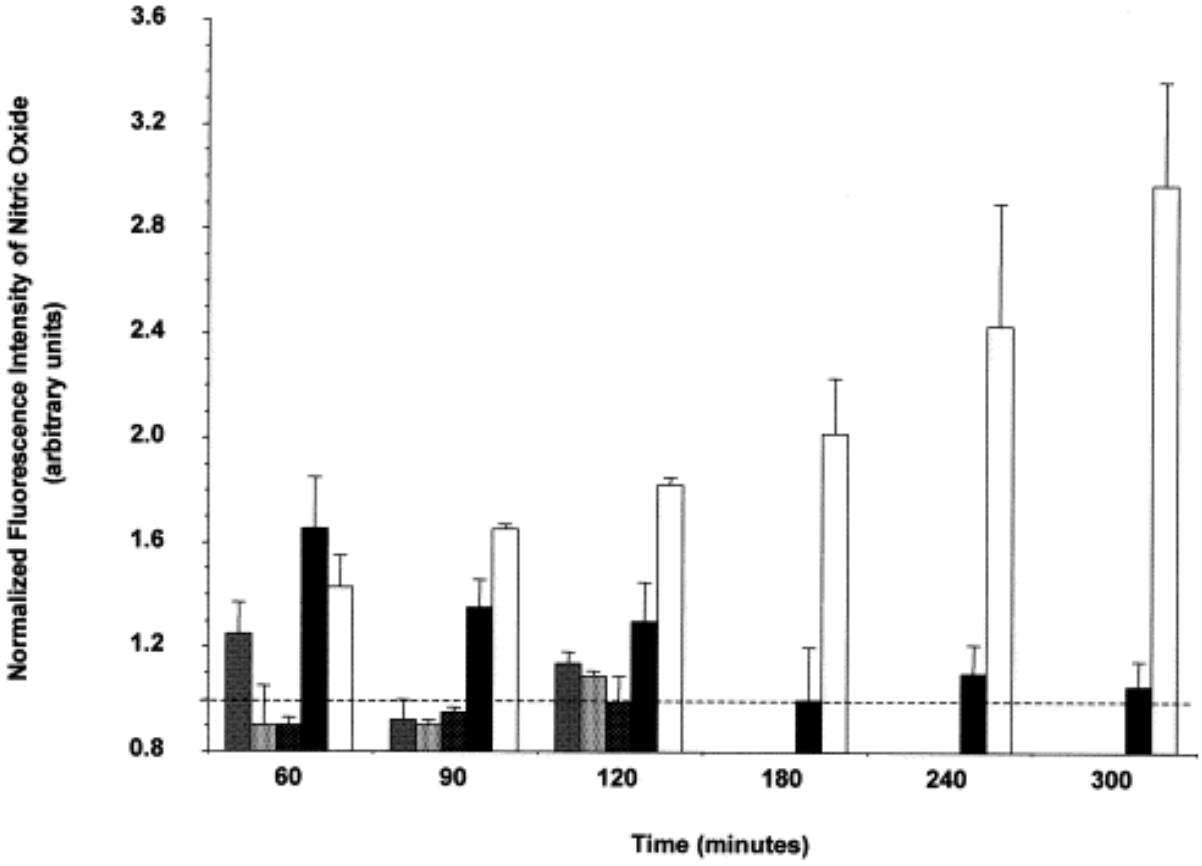

Figure 10. – Human saphenous vein graft segments stored in different preservation solutions to measure NO generation by incubation in diaminofluorescein (DAF).

Human saphenous vein graft segments stored in different preservation solutions before incubation in diaminofluorescein (DAF) to measure NO generation: Intensity of DAF at the endothelial layer was measured via multiphoton microscopy after 10 minutes of treatment with bradykinin. NO generation acutely decreased in segments stored in heparinized lidocaine saline (shaded bars), autologous heparinized blood (black-spotted bars), and tissue culture medium (white-spotted bars). Segments stored in Hank's balanced salt solution (black bars) underwent a progressive decrease in NO generation after being maintained in vessels stored for shorter periods. Conversely, segments stored in GALA (white bars) showed a well-preserved eNOS activity and NO generation. In fact, a sustained increase in NO production was observed as the period of storage in GALA was extended [184]. *Permission to reuse obtained (Annex 1)*

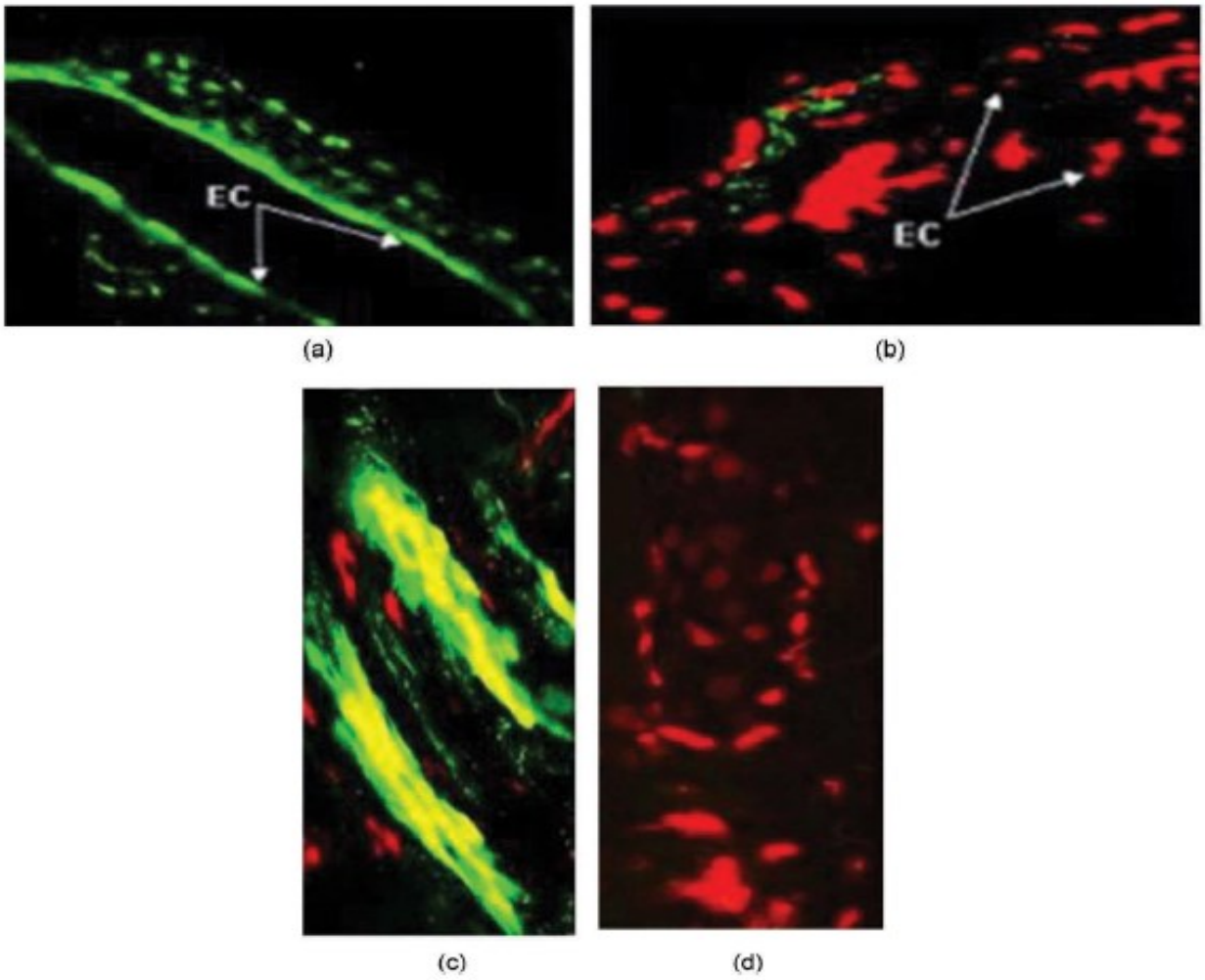

Figure 11. – Multiphoton imaging of human saphenous vein stored in GALA for 1 hour (a) and saline for 1 hours (b), for 15 min (c) and 30 min (d).

Green fluorescence represents viable cells and red fluorescence indicates nonviable cells while the yellow staining designates a blend of viable and nonviable cells. HSV segments display extensive cell mortality and loss of structural integrity as early as after 15 min exposure in saline (c). HSV segments stayed well preserved in terms of their structure and cell viability even after 1 hour of exposure to GALA (a) [192]. *Open access article - no permission required (Annex 1).*

3.4- Vascular reactivity study with GALA solution

In 2016, Wise and colleagues published a study in which vascular reactivity studies were performed in organ chambers experiments to assess the endothelial function of HSV conduits in the presence of various solutions including GALA. This technique is the same as the one used in the current study. Organ chambers are used to measure physiological responses such as contraction and relaxations of blood vessels in response to drugs. Harvested blood vessels are divided into rings and hung to metal stirrups inside organ baths. Each individual organ bath mimics physiological conditions in terms of oxygenation, temperature, and are filled with prepared physiological solution in which the rings are submerged. Upon the addition of an agonist to the organ bath, the rings contract or relax and thus applying a mechanical force to the hook. The mechanical force is transferred to a transducer that converts into an electrical force. The signal is amplified and relayed to a computer storing variations in isometric tensions for each ring enabling the construction of dose-response curves generated by gradually increasing a given agonist's concentration (Figure 12).

Wise et al compared the effects of six solutions on the endothelial function of HSVs after 2-hours storage. The six solutions are Plasma-Lyte A, 0.9% NaCl (normal saline), University of Wisconsin solution, Celsior solution, autologous whole blood, and GALA [197]. The control group consisted of unprepared (UP) also labelled as unmanipulated (UM) vessels that were minimally handled and were hung in organ baths directly after harvesting. The study reported that saline resulted in impaired contractility to KCl and phenylephrine. Evaluation of endothelium-dependent relaxations is a sensitive way to assess whether endothelial damage occurred during storage. Percentage of relaxation is determined by the percentage decrement from phenylephrine-generated contraction. Carbachol (CCh), a receptor and endothelium-dependent relaxant was used across all groups to compare responses and evaluate whether the endothelial function was preserved. Sodium nitroprusside (SNP), an endothelium independent relaxant which directly diffuses to the smooth muscle to cause relaxation, is used to validate the integrity of the smooth muscle. HSV preserved in saline showed impaired smooth muscle responses to nitric oxide donor SNP and to the cholinomimetic inducer of endothelial NO production CCh. Compared to the unmanipulated

control group, all solutions led to decreased endothelium-dependent relaxations except AWB and GALA (figure 13).

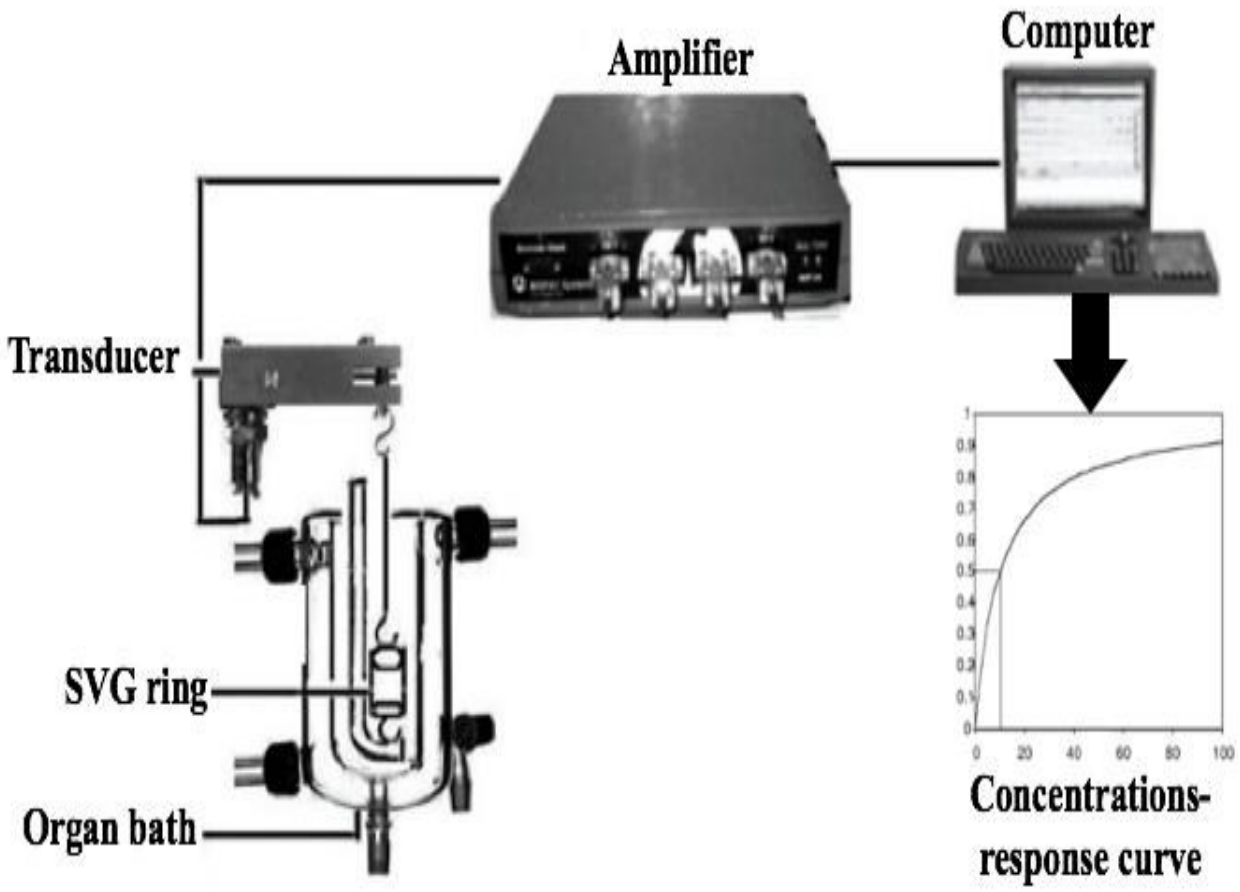

Figure 12. – Diagram of isolated organ chamber preparation used to record physiological tissue responses to drug concentrations.

Use of diverse drugs/agonists enables the investigation of different cellular pathways including the endothelial reactivity function. Agonists are added to organ baths filled with Krebs-bicarbonate solution in which SVG rings are suspended between two metal stirrups placed through the lumen and connected to an isometric force transducer. *Adapted from Ocal I et al [207].*

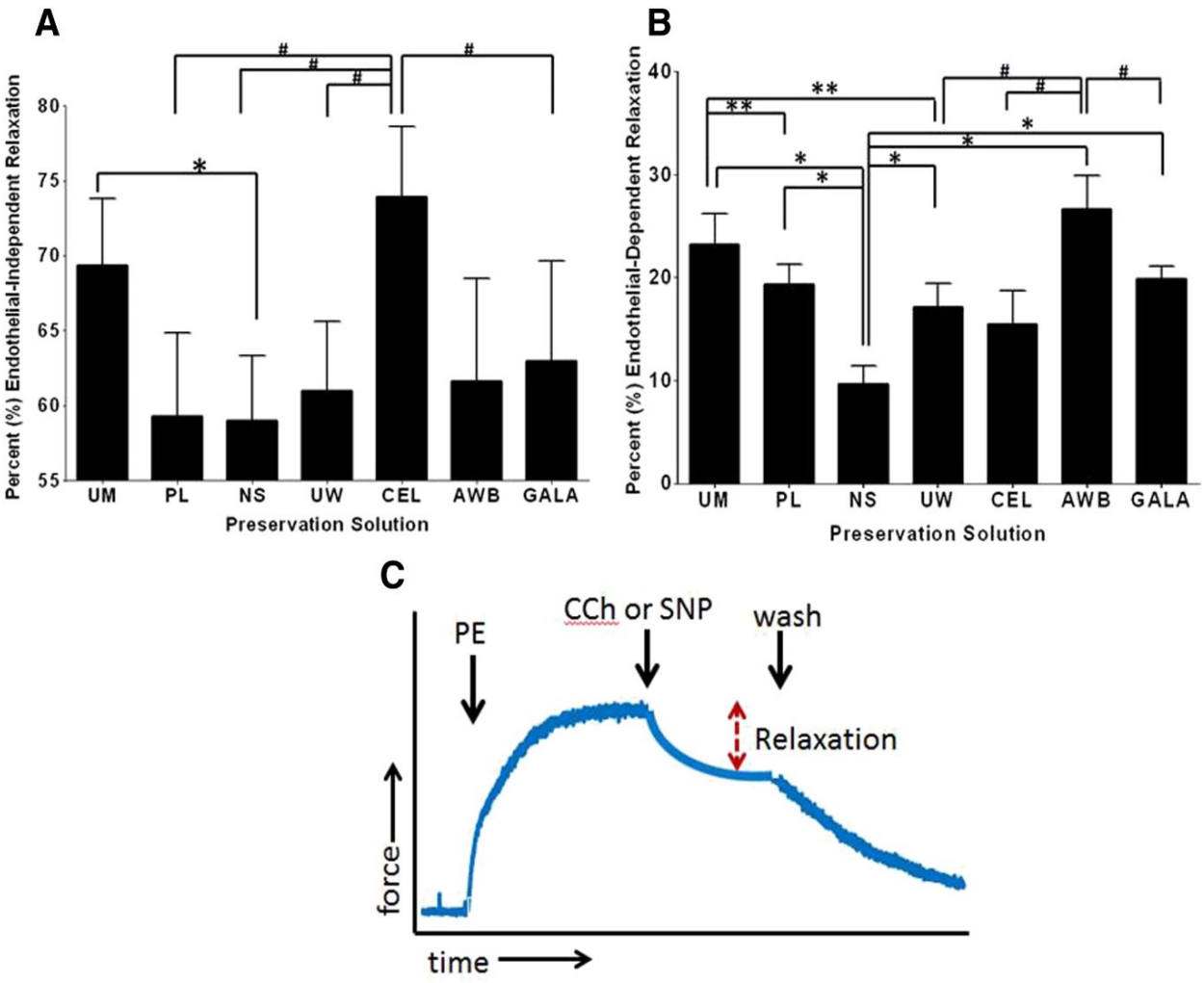

Figure 13. – Relaxation responses of human SVs (HSVs) in various solutions and a control group (UM) post exposure to agonists.

Unmanipulated (UM), Plasma-Lyte A (PL), normal saline (NS), University of Wisconsin (UW), Celsior (CEL), Autologous whole blood (AWB), GALA. A. Endothelium-independent smooth muscle relaxation of HSVs in response to NO donor sodium nitroprusside (SNP). B. Endothelium-dependent smooth muscle relaxation of HSVs in response to Carbachol (CCh).C. Sketch representing relaxation response versus time in organ chambers[197]. *Permission to reuse obtained (Annex 1).*

Fouquet and colleagues published a study investigating the endothelial function of arterialized vein grafts in an experimental rat model in 2020 [208]. In that study GALA did not reduce the risk of intimal hyperplasia upon exposure of rat vein graft to arterial flow. A strongly worded letter to the editor from several researchers argued that the solution to which Fouquet referred to as GALA in their study had not the same composition as GALA (that was presented in the previously discussed articles above) and therefore was not comparable to DuraGraft® [209]. In fact, Fouquet's pseudo "GALA solution" has no buffering capacity and had an ascorbic acid concentration 23-fold higher (11.4 mM) than Thatte's original GALA mixture (0.5 mM). These extremely high concentrations of ascorbic acid used by Fouquet's are cytotoxic and acidic even if a basic component such as sodium bicarbonate was added.

3.5- Clinical studies investigating GALA:

Up to now, only one double-blinded, randomized controlled clinical trials evaluating the efficacy of different preservation solutions was conducted [210]. However, two clinical studies investigating the clinical outcome on patients have been published. In the retrospective study published in the journal Expert Review of Cardiovascular Therapy, Haime et al compared the clinical results of 2463 patients undergoing isolated CABG with DuraGraft® and 1400 patients with saline to evaluate the short (<30 days) and long-term (≥ 1000 days) outcomes using reoperation as the primary end point as well as major adverse cardiac events (MACE defined as death, nonfatal myocardial infarction, or repeat revascularization)[211]. For patients whose SVGs were stored in DuraGraft® before implantation, nonfatal myocardial infarctions were significantly lower by 45% while repeat revascularization significantly diminished by 35% in patients (Figure 14) [211].

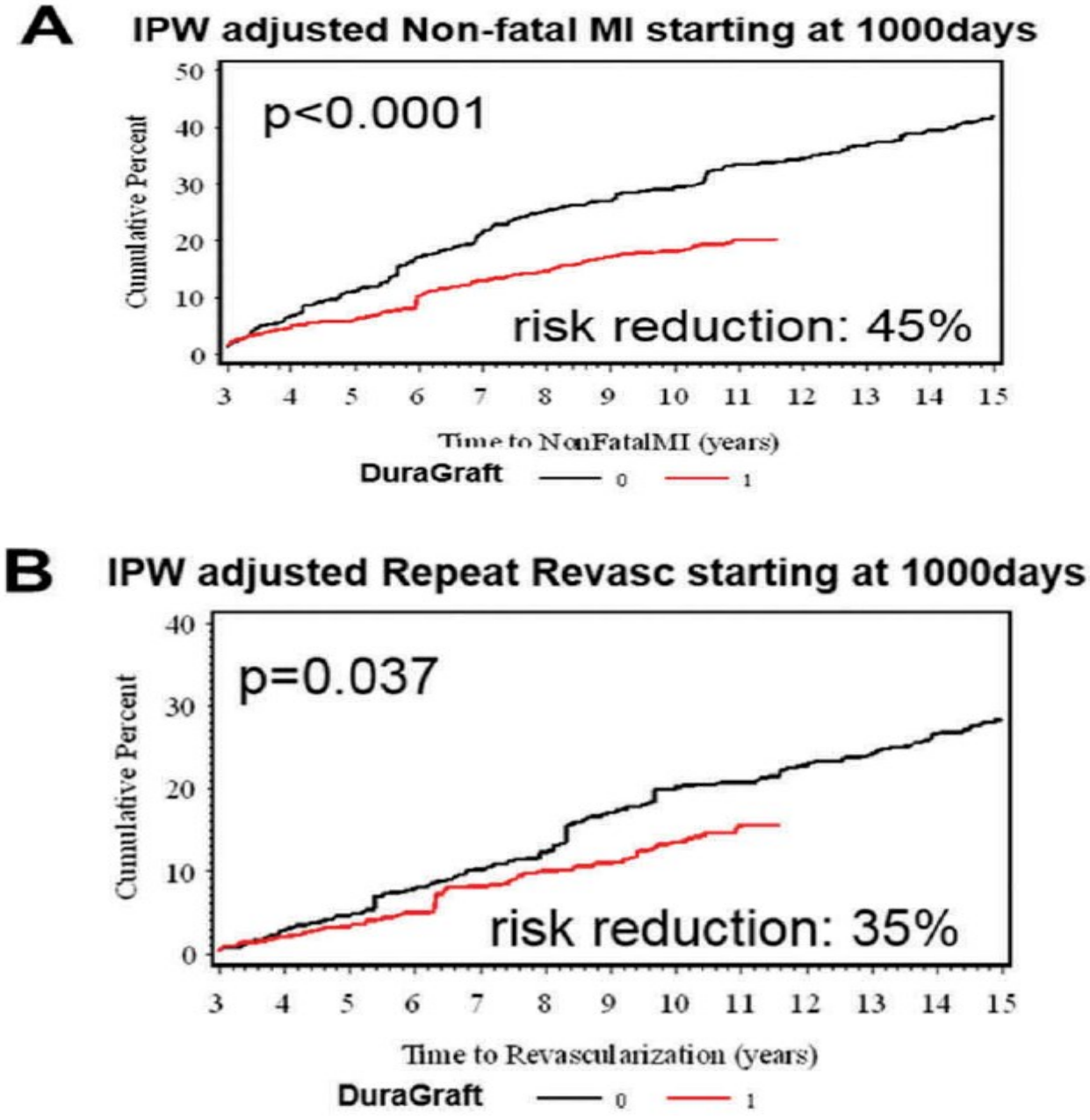

Figure 14. – Cumulative percentage of non-fatal myocardial infarction and repeated revascularization starting at 1000 days post CABG.

Both non-fatal myocardial infarction (a) and repeat revascularization were significantly lower in the patients whose SVGs were treated with DuraGraft®[211]. IPW: inverse probability of treatment weight [211]. *No permission required for use in dissertation/thesis (Annex 1).*

Another ongoing clinical study by a European group still currently enrolling aims to recruit 3000 patients undergoing isolated CABG procedure or a combined procedures with at least one SVG or one free arterial graft (radial or mammary artery) [212]. All free grafts will be stored and treated with DuraGraft®. Primary outcomes will be assessed by looking at the incidence of MACE (Death, nonfatal myocardial infarction, repeat revascularization). Secondary outcomes are the incidence of major adverse cardiac and cerebrovascular events (MACCE is defined as death, nonfatal myocardial infarction, repeat-revascularisation, or stroke). The patient's quality of life and health-economic data will also be recorded. Data will be collected during the patient's hospital stay, at 1-month, 1-year, and each year until 5 years post-CABG. For now, the results are still not published for this study.

This present *ex-vivo* sub-study stems from the multicenter clinical study conducted at the Montreal Heart Institute under the supervision of the same principal investigator and cardiovascular surgeon, Dr. Louis P. Perrault. The clinical trial was prospective, multicenter, randomized, double-blind, comparative within patient study for 119 patients [213]. Perrault and colleagues compared the effects of GALA versus heparinized saline in patients requiring at least two SVGs when undergoing isolated first-time CABG for multivessel coronary disease. Follow-up with multidetector computed tomography (MDCT) at 1-month, 3 months, and 1 year was performed to observe the between groups difference in wall thickness, lumen diameter, and maximum narrowing [214]. At 3 months, no significant changes were observed between both groups for wall thickness, lumen diameter and maximum narrowing. At 12 months, GALA-treated grafts had significantly lower mean wall thickness while the maximum graft narrowing of saline-treated grafts was larger. Moreover, occlusion or thrombosis occurred in eleven saline-treated graft versus nine in GALA [214]. In total, 20 SVGs (9 GALA [7.2%] and 11 saline [8.8%]) were occluded and the majority (n =19) of the thromboses or occlusions were observed at the 3-month MDCT [214].

The objective of the present sub-study is to evaluate the functional integrity of the endothelium of saphenous vein grafts following storage in GALA vs heparinized saline using ex vivo vascular reactivity studies. To assess the vascular reactivity, SVGs will be exposed to increasing doses of acetylcholine (ACh), (endothelium-dependent relaxant agonist of M_2 receptors

coupled to G_i-proteins leading to release of nitric oxide resulting in vasodilation) , and the calcium ionophore A23187 (receptor-independent endothelium-dependent relaxant agonist leading to release of nitric oxide and endothelium-derived hyperpolarizing factor). Sodium nitroprusside, an exogenous nitric oxide donor will be used to ensure the integrity of underlying vascular smooth muscle cells. The main hypothesis is that GALA-treated SVGs will exhibit superior endothelium-dependent relaxations since it contains additives to protect and enhance endothelial function compared to heparinized saline.

Chapter 4 – Material & Methods

4.1-Study design

This study was part of a clinical trial designed as a prospective, multicenter, randomized, double-blinded, and comparative within patient study to evaluate the use of GALA one-time, intraoperative graft treatment versus heparin dosed saline (standard of care) in patients undergoing isolated CABG.

4.1.1- Patient population

Eligible patients include those aged between 18 and 80 years undergoing primary and isolated, multi-vessel CABG with at least two SVGs. Patients with previous heart surgery, poor left ventricular ejection fraction define, other significant valve disease, need for aortic aneurysm repair, with a stroke or transient ischemic attack within the previous 12 weeks or any contraindication to cardiopulmonary bypass were excluded.

4.1.2- Randomization

Randomization was ensured when placing the segments in the solutions as segments were randomly assigned to each container. The surgeon was blinded to the preservation and storage solutions used for the two SVGs in each enrolled patient. Since GALA is a transparent solution; it was indistinguishable from the standard of care (heparinized saline).

4.2-Preservation solution

DuraGraft® (SOMVC001) is a specifically designed one-time intraoperative treatment to protect against damage to the structure and function of the vascular endothelium. GALA is formulated into an ionically and pH-balanced physiological salt solution containing electrolytes (sodium, chlorine, potassium, magnesium, calcium, bicarbonate and sulfate) and other ingredients (Glutathione, L-Ascorbic Acid, and L-Arginine (GALA)), aimed at protecting the conduit from the damaging

effects of ischemia (during storage) handling and reperfusion during CABG. GALA was compared with the standard of care (SOC) solution, heparin dosed saline, used at our institution for the preservation, storage and flushing of vascular conduits.

4.3-Graft Preservation and storage

The SVG was harvested using state-of-the-art harvesting (open or endoscopic) and handling techniques (i.e. atraumatic surgical technique, avoidance of over pressurization during checking for leakage, excessive handling and distortion) in order to reduce traumatic damage to the conduit endothelium. The SVG segment was divided into two equal segments that were carefully flushed with and stored emerged in the assigned solution. Storage time from storage to anastomosis was recorded for each of the conduits with a minimum duration of at least 15 minutes. The SVG segments were only exposed to the assigned preservation solution from the time of harvest until the beginning of the *ex vivo* experiments.

4.4-GABG procedure

CABG surgery either using on-pump or off-pump techniques (at the discretion of the surgeon) was performed according to standard surgical techniques at the institution.

4.4.1- *Ex Vivo* evaluation of saphenous venous segments

Ex vivo laboratory assessments were conducted in a subset of patients undergoing CABG at the Montreal Heart Institute. To conduct these assessments, remaining stored vein segments from each of the storage solutions were immediately sent to the laboratory functional evaluations according to a pre-established procedure. The time interval between explantation and placement in the organ baths to be oxygenated and examined did not exceed 1 hour [215].

4.5- Assessment of endothelial function

4.5.1- Vascular reactivity studies

To evaluate vascular reactivity of the SVGs, organ chamber experiments were performed based on a modified protocol previously described in literature [215-217]. Briefly, SVG segment were divided into 3 mm wide rings and suspended between two metal stirrups connected to an isometric force transducer in isolated organ chambers containing modified Krebs-bicarbonate solution (20 mL) maintained at 37°C, and oxygenated with a carbogen gas mixture (95% oxygen/5% carbon dioxide). Data were recorded using the data acquisition and analysis software IOX (version 1.6, Emka Technologies, Paris, France).

4.5.1.1-Contractions

After 30 minutes of stabilization, rings were gradually stretched to their optimal tension (approximately 1.5-2 g) [216] and then allowed to stabilize for another 90 min. This value was previously determined by measuring the contractile response to potassium chloride (KCl; 90 mM) at increasing levels of stretch corresponding to the optimal point on the active length-tension curve. The maximal contraction was determined with KCl (90 mM) and rings were excluded if they failed to contract (exclusion rate <5%). After 45 minutes of stabilization, a pre-contraction to phenylephrine (PE) was conducted to achieve 50-80% of the maximal contraction observed with KCl (90 mM) [197].

4.5.1.2-Endothelium-dependent relaxations

Endothelium-dependent NO-mediated relaxations were studied by characterizing concentration-response curves to acetylcholine (ACh; 10^{-9} to 10^{-4} M; an agonist of M_2 receptors coupled to G_i-proteins, leading to release of NO) and to calcium ionophore A23187 (10^{-9} to 10^{-5} M; receptor-independent, leading to release of NO and endothelium-derived hyperpolarizing factor

(EDHF). In the course of the experiments, no rings were exposed to more than one endothelium-dependent agonist.

4.5.1.3-Endothelium-independent relaxations

Endothelium-independent relaxations were evaluated with incremental concentrations of sodium nitroprusside (SNP; 10^{-10} to 10^{-5} M; exogenous NO donor), allowing to assess the integrity (preserved relaxing ability) of underlying smooth muscle cells.

4.6-Statistical analysis

Endothelium relaxations were expressed as a percentage of the maximal contraction to PE and presented as mean ± standard error of the mean (SEM). Maximal response (E_{max}), half-maximal effective concentration (EC_{50}) and negative logarithm to base 10 of the EC_{50} (pEC_{50}) were determined from each patient's concentration-response curve (sigmoidal dose-response (variable slope); equation ($Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC50 - X) \cdot HillSlope}}$) using the curve-fitting software GraphPad Prism (Version 9.1.2, GraphPad Software, La Jolla, CA, USA). For these parameters, differences between groups were evaluated with a 2-tailed paired student *t* test. Results were presented as mean ± SEM of n patients and a *P*-value <0.05 was considered statistically significant.

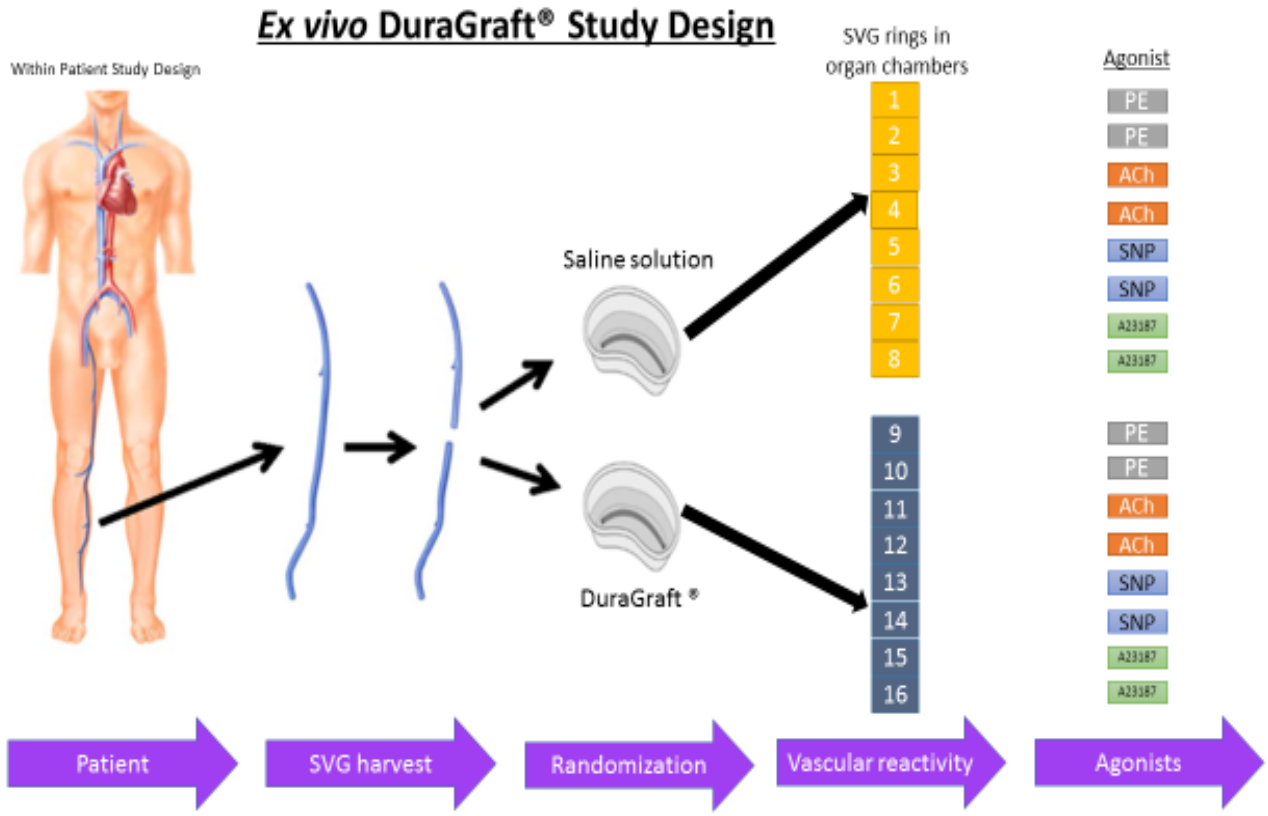

Figure 15. – ***Ex vivo*** **GALA/DuraGraft® study design.**

After SV harvesting, one segment is placed in saline and the other in GALA/DuraGraft®. Segments are cut into rings and placed in organ chambers in which each ring is exposed to one agonist. Sketch assumed a maximum capacity to use 16 organ chambers (the number of rings and baths used in each experiment depends on the available and usable length of the SVGs). When the number of rings was limited, selected agonists were applied to all 15 patients. In some cases, more than agonist was applied to one ring (ACh followed by SNP) but never two endothelium-dependent relaxing agonists. SNP and A23187 were always the final agonists applied to a ring that received more than one agonist since rings are rendered unreactive after their application.

Chapter 5 – Results

5.1-Endothelium-independent contraction

Smooth muscle cell contraction functionality was first tested before assessing endothelial function. KCl-induced contractions were used to ensure that the rings were reactive after surgical manipulations in the operation room and after handling in the laboratory. There were no statistically significant differences in the amplitude of maximal contraction to potassium chloride (GALA 9.9 ± 0.9(g); Saline 8.4 ± 0.9(g)) (Table 2). The concentration of PE required to reach the target contraction of 50-80% was not significantly different in both groups (GALA 0.07 ± 0.01; Saline 0.20 ± 0.13) (Table 2). The resulting PE- induced contractions were 72.5% ± 2.0 and 73.9% ± 1.8 for GALA and saline, respectively (Table 2). pEC_{50} (GALA 5.22 M ± 5.41; Saline 4.98 M± 5.34) and Emax values (GALA 124.9 % ± 10.9, Saline 137.2% ± 19.5) of PE concentration-response curve showed no significant difference (P>0.05) in both groups (Table 3 and Figure 16). Overall, there were no statistically significant differences in the amplitude of the contractions to KCl and to phenylephrine in both GALA and saline groups.

Tableau 2. – Contractions of HSV to potassium chloride (KCl; 90 mM) and to phenylephrine (PE) after storage in GALA vs. Saline

	100% contraction using KCl (90 mM)	[PE] required to reach 70-80% of KCl contraction	PE
	g	g	% KCl 90 mM
GALA	9.9 ± 0.9	0.07 ± 0.01	72.5 ± 2.0
Saline	8.4 ± 0.9	0.20 ± 0.13	73.9 ± 1.8

Data are expressed as mean ± SEM of n patient. KCl, potassium chloride; PE, phenylephrine. GALA n=15 patients; Saline n=15 patients. *P*>0.05; GALA vs. saline

Tableau 3. – pEC_{50} and Emax values of HSV concentration-response curves to Phenylephrine (PE) after storage in GALA vs. Saline

	pEC_{50} (M)	Emax (%)
	PE	PE
GALA	5.22 ± 5.41	124.9 ± 10.9
Saline	4.98 ± 5.34	137.2 ± 19.5

Data are expressed as mean ± SEM of n patients. Emax, maximal response; pEC_{50}, negative logarithm to base 10 of the half maximal effective concentration; PE, phenylephrine; GALA n=5 patients; Saline n= 5 patients. *P*>0.05; GALA vs. saline.

Contraction in Response to PE of HSVs Stored in GALA vs Saline

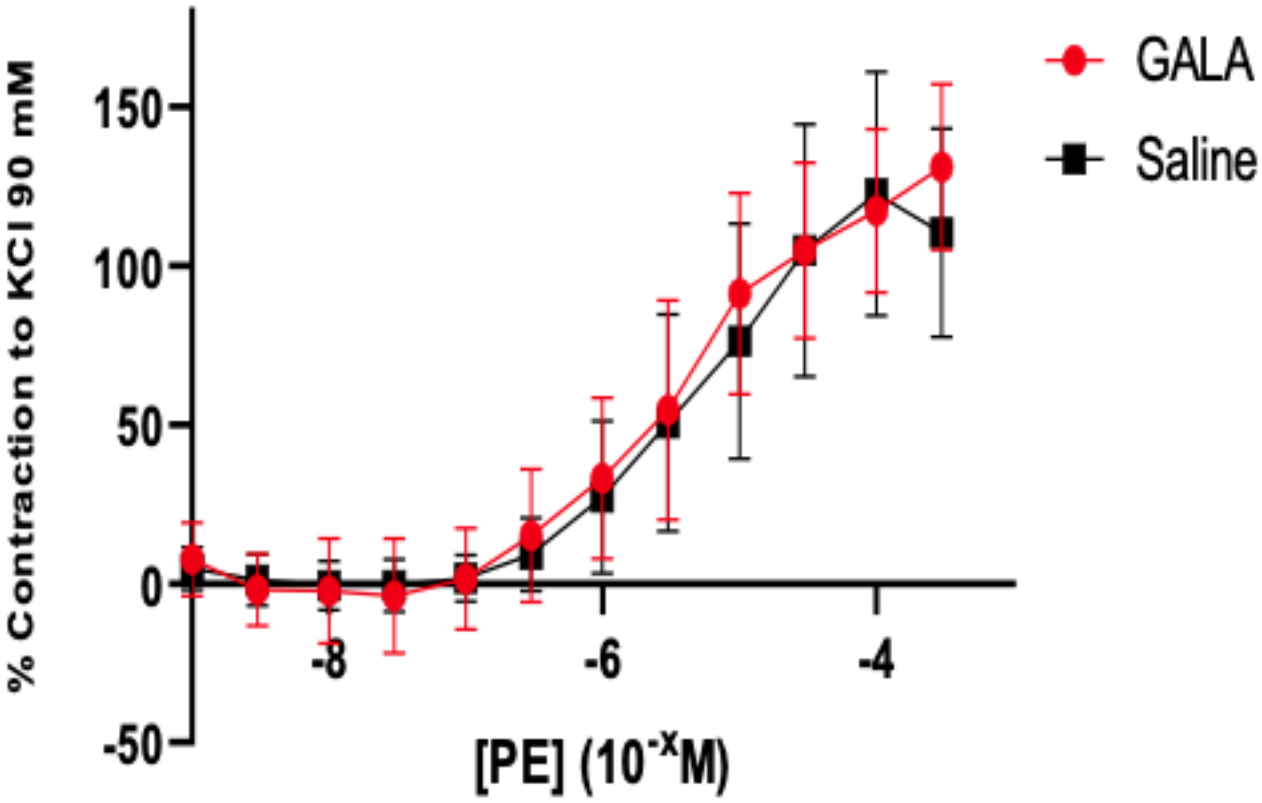

Figure 16. – Contractions: cumulative concentration-response curves in response to phenylephrine (PE) in rings of human saphenous vein after flushing and storage within GALA (●) n=5 patients and saline (■) n=5 patients; preservation solution in patients undergoing coronary artery bypass grafting (CABG). Responses are expressed as the percentage of maximal contraction induced by potassium chloride (90 mM). Results are presented as mean ± SEM of n patients. *P*>0.05; GALA vs. Saline.

5.2-Endothelium-independent relaxations

Sodium nitroprusside (SNP) is an exogenous NO donor, that relies on the NO produced by eNOS to induce SM relaxation. Instead, SNP directly diffuses in the underlying SMCs to cause endothelium-independent relaxations. Responses to SNP represent a functional assessment of smooth muscle tissue exclusively. HSV rings preserved in GALA exhibited significantly greater endothelium-independent relaxations relative to preservation in saline (P<0.00001) (Figure 17). pEC50 (GALA 6.74 ± 6.95; Saline 6.69 M± 6.94) and Emax values (GALA-149.6 %± 18.1; Saline -139.7%± 11.3) values of HSV SNP concentration-response curves showed no statistically significant differences between both groups (Table 4).

Tableau 4. – pEC_{50} and Emax values of HSV concentration-response curves to SNP after storage in GALA vs. Saline

	pEC_{50} (M)	Emax (%)
	SNP	SNP
GALA	6.74 ± 6.95	-149.6 ± 18.1
Saline	6.69 ± 6.94	-139.7 ± 11.3

Data are expressed as mean ± SEM of n patients. Emax, maximal response; pEC_{50}, negative logarithm to base 10 of the half-maximal effective concentration; SNP, sodium nitroprusside. GALA n= 7 patients; Saline n= 7 patients; GALA vs. Saline. *P*>0.05.

Endothelium-independent Relaxation in Response to SNP of HSVs Stored in GALA vs Saline

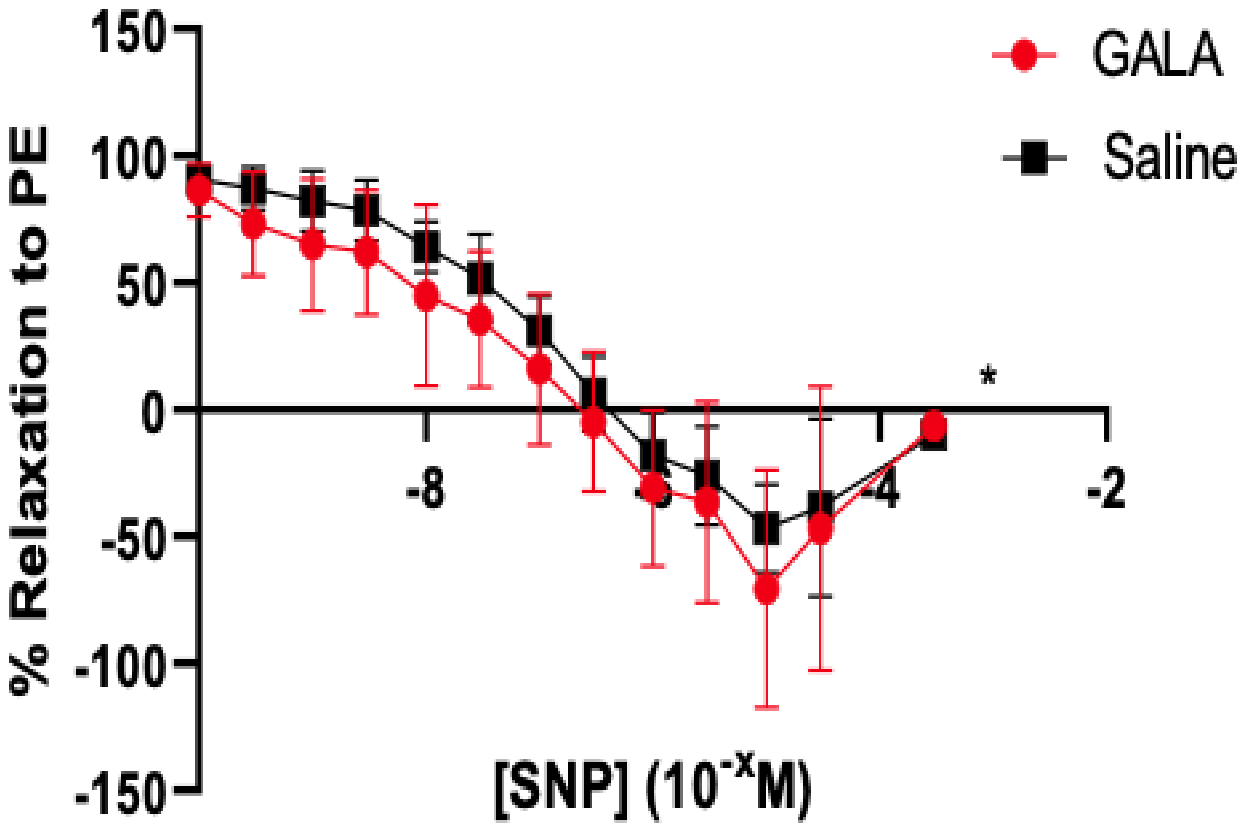

Figure 17. – Endothelium-independent relaxations: cumulative concentrations-response curves in response to sodium nitroprusside (SNP) in rings of human saphenous vein after flushing and storage within GALA (●) n=7 and saline (■) n=7 patients; preservation solution in patients undergoing coronary artery bypass grafting (CABG). Responses are expressed as the percentage of relaxation to contraction induced by phenylephrine (PE). Results are presented as mean ± SEM of n patients. **P*<0.00001; GALA vs. saline.

5.3-Endothelium-dependent relaxations

Endothelium-dependent relaxations were assessed to determine if the vasorelaxant tone was preserved and if the development of an altered vascular reactivity was prevented in HSV stored in the experimental (GALA) and control solutions (Saline). Endothelium-dependent relaxations were studied by characterizing concentration-response curves to ACh and the calcium ionophore A23187. ACh endothelium-dependent relaxation are initiated by an action of this agonist on M2 receptors coupled to Gi-proteins of endothelial cells. The action of ACh on these receptors triggers calcium release from the internal store in endothelial cells to stimulate the production of relaxants factors such as nitric oxide, which then acts on smooth muscle cells to cause vasorelaxations. The calcium ionophore A23187 generates endothelium-dependent and receptor-independent relaxations since this ionophore transport Ca2+ directly across endothelial cells membrane fully activating the mechanism for production of NO and other endothelium-derived hyperpolarizing factor. We found a statistical significant decrease of endothelium-dependent relaxations in the saline treated group in response to both ACh and A27187 upon comparison to HSV rings preserved in GALA (ACh $P<0.01$ (Figures 18); A23187 $P<0.0001$(Figure 19)). The difference in pEC50 (ACh: GALA 5.67 M ± 5.68; Saline 6.32 M± 6.37; A23187: GALA 6.97 M± 7.19; Saline 6.95 M ± 7.14) and Emax values (ACh: GALA 54.8 % ± 5.1; Saline 44.1%± 8.3; A23187: GALA, 41.5 %± 7.94; Saline 35.0 %± 14.6) did not reach statistical significance, Relaxation of isolated HSV rings exposed to saline was significantly impaired (Table 5). This is demonstrated by an upward shift in ACh and A23187 concentration-response curves of the saline-treated group showing decreased endothelium-dependent relaxations (Figures 18 and 19).

Tableau 5. – pEC_{50} and Emax values of HSV concentration-response curves to ACh and A23187 after storage in GALA vs. Saline

	pEC_{50} (M)		Emax (%)	
	ACh	A23187	ACh	A23187
GALA	5.67 ± 5.68	6.97 ± 7.19	54.8 ± 5.1	41.5 ± 7.94
Saline	6.32 ± 6.37	6.95 ± 7.14	44.1 ± 8.3	35.0 ± 14.6

Data are expressed as mean ± SEM of n patients. ACh, acetylcholine; A23187, calcium ionophore A23187; Emax, maximal response; pEC_{50}, negative logarithm to base 10 of the half maximal effective concentration. GALA: ACh n=8 patients, A23187 n= 5 patients; Saline: ACh n= 8 patients; A23187 n= 3. $P>0.05$; GALA vs. Saline.

Endothelium-dependent Relaxation in Response to ACh of HSVs Stored in GALA vs Saline

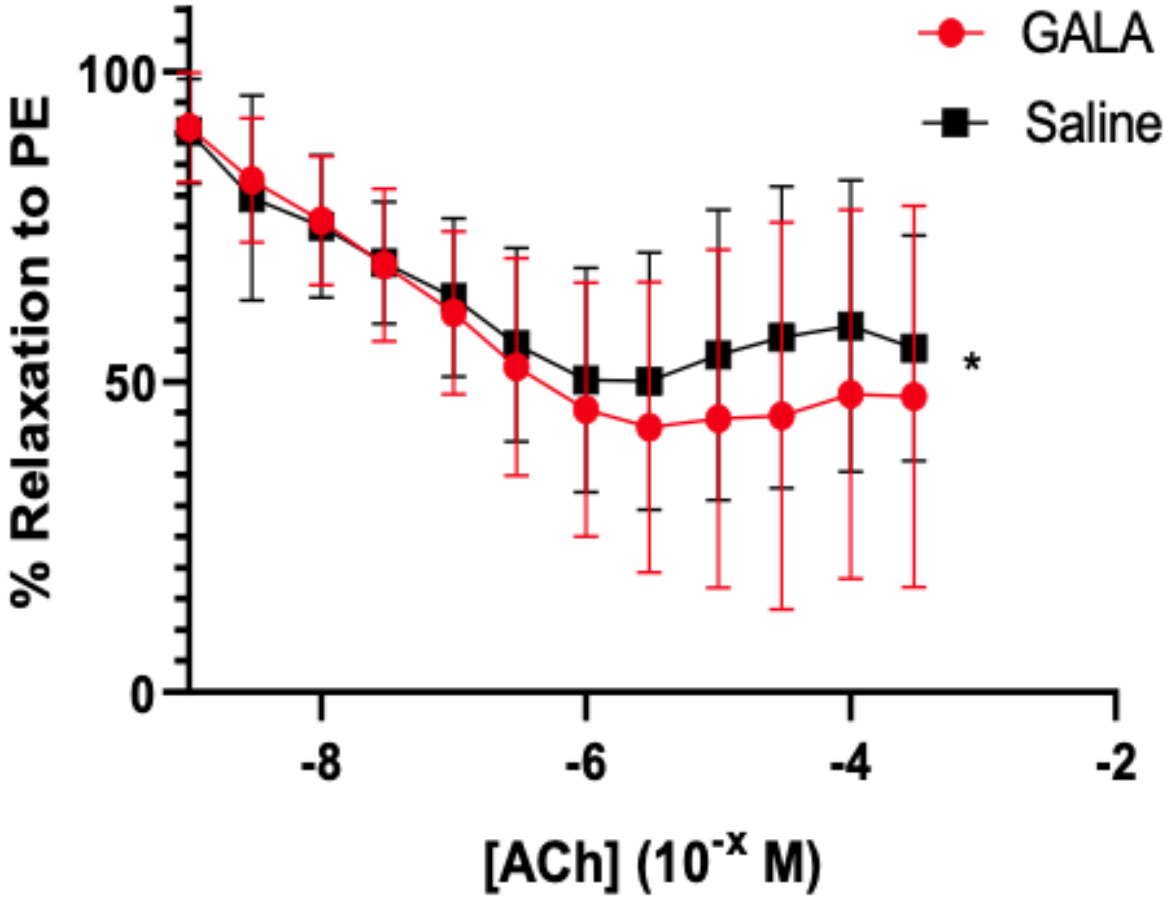

Figure 18. – Endothelium-dependent relaxations: cumulative concentration-response curves in response to acetylcholine (ACh) in rings of human saphenous vein after flushing and storage within saline (■) n= 10 patients; and GALA (●) n= 10 patients; preservation solution in patients undergoing coronary artery bypass grafting (CABG). Responses are expressed as the percentage of relaxation to contraction induced by phenylephrine (PE). Results are presented as mean ± SEM of n patients. **P*<0.01; GALA vs. saline.

Endothelium-dependent Relaxation in Response to A23187 of HSVs Stored in GALA vs Saline

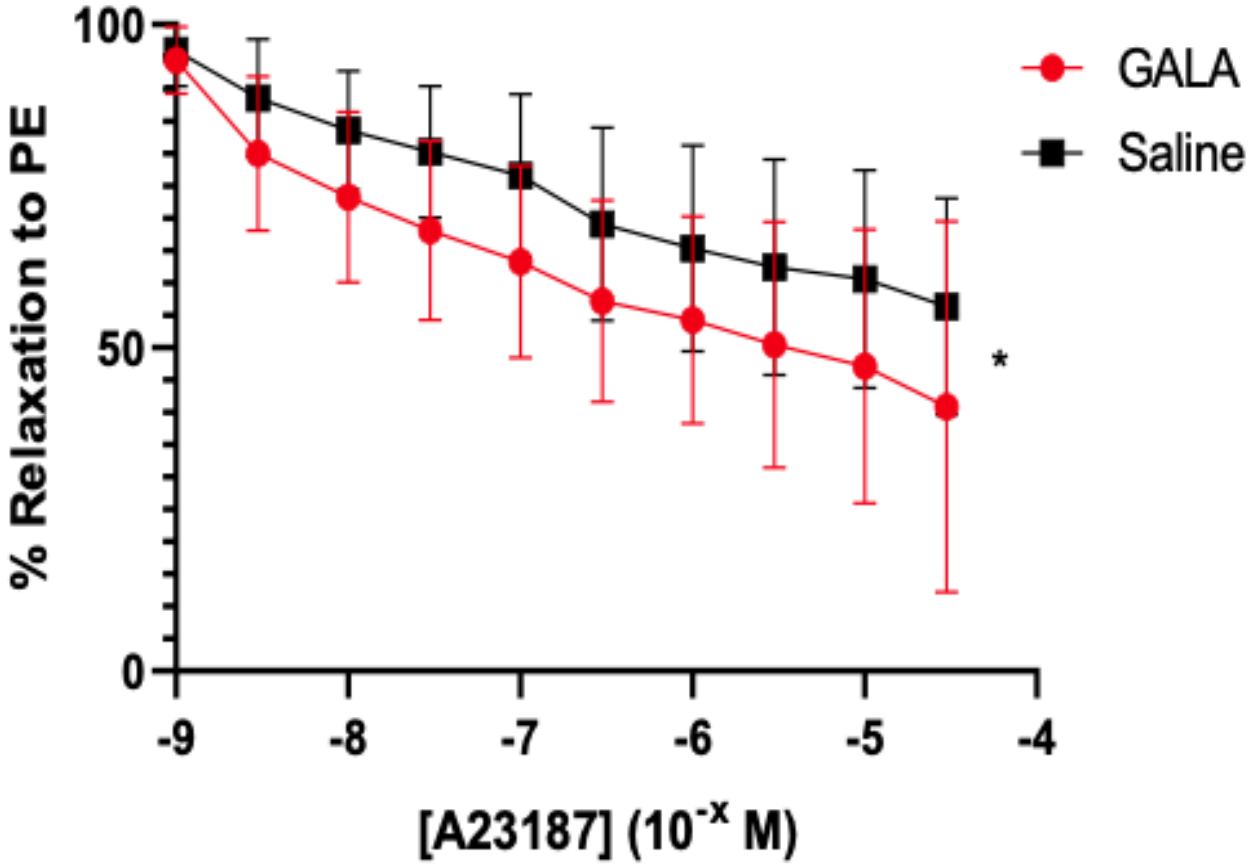

Figure 19. – Endothelium-dependent relaxations: cumulative concentrations-response curves in response to the calcium ionophore A23187 in rings of human saphenous vein after flushing and storage within saline (■) n=11 patients; and GALA (●) n=11 patients; preservation solution in patients undergoing coronary artery bypass grafting (CABG). Responses are expressed as the percentage of relaxation to contraction induced by phenylephrine (PE). Results are presented as mean ± SEM of n patients. **P*<0.0001; GALA vs. Saline.

Chapter 6 – Discussion

The major finding of the present study is that the GALA solution preserves the endothelial function in HSV rings during storage and cold ischemic time. Because cellular viability correlates with functional viability in HSV[165], our results indicate foremost that heparinized saline preservation promotes HSV cellular injury. Despite the same amplitude of contractions in response to KCl and PE in both groups, impaired smooth muscle responses were observed in the saline treated group not only upon exposure to exogenous NO donor sodium nitroprusside, but also upon treatment with the two endothelium-dependent relaxants agonists, acetylcholine and calcium ionophore A23187.

The negative impact of vessel storage in normal saline were first brought forward by O'Connell and al in 1974 when increased myointimal proliferation in rabbit carotid arteries preserved in NS was observed [218]. One of normal saline biggest drawbacks is its acidic pH. Wise et al demonstrated how storage at a physiological pH is critically important to maintain functional integrity and cellular viability of HSV when they compared endothelial function in rat aortas preserved at different pH values by carbachol challenge. NS (pH 5.9) displayed the greatest endothelial functional damage with decreased endothelium-dependent smooth muscle relaxations response to carbachol compared to HSVs stored in Plasmalyte-A which has a physiological pH (pH 7.0-7.4) [197]. Because saline is not buffered, ambient carbon dioxide causes its acidification leading to a pH < 6 [197]. In a clinical study, Harskamp and colleagues investigated the effect of pH by preserving HSVGs in saline, blood or buffered saline solutions on the outcome of SVG after CABG by performing systematic one-year angiography examine VGF along with a 5-year follow-up period. SVG preserved in buffered saline solutions showed greater vein graft patency rates and trends towards improved clinical outcomes at 5-years while SVG preserved in saline had both the highest percentage of patient-level VGF and graft-level VGF [169].

Lacking electrolytes and endothelial protective additives, NS does not mitigate oxidative damage and metabolic stress mediating ischemic injury but instead promotes leukocytes and thrombocyte adhesion leading to the development of graft atherosclerosis[219]. Indeed, Thatte et al 2003 and Pachuk et al 2019 both published similar results showing greater damage to endothelial structural integrity and decreased cell viability in HSVs stored in NS[184]. Taking into account the acidic pH, the lack of biocompatibility, the mild hyperosmolarity, and the absence of cellular nutrients and antioxidants, impairment of both endothelial functions and structural integrity, strong recommendations were emitted to stop using normal saline to preserve HSVGs [200]. Injury to SVG during harvesting and endothelial dysfunction resulting from the type of preservation solution used during cold ischemia, promote thrombogenesis and neointimal hyperplasia that can progress to atherosclerosis characterized by SVG stenosis and occlusion. [158, 170] [169]. Damages to endothelial structure and the ensuing dysfunction are amongst the main reasons for progression to VGF [211]. These factors are synergistic in speeding up the development of intimal hyperplasia, which may progress to thrombosis and can to lead to sudden death or graft failure after initial CABG surgery. Recent clinical studies found that storage of human SVGs in GALA was associated with lower risk of long-term adverse events suggesting that an efficient intraoperative SVG treatment may reduce VGF related complications post-CABG [211, 213].

Our working hypothesis was that GALA would protect HSVs endothelial function and provide superior endothelium-dependent smooth muscle relaxations due to its specific design for vascular conduit preservation. GALA contains endothelial protective additives such as glutathione and L-ascorbic acid, antioxidants, and arginine (the substrate for nitric oxide synthase in endothelial cells). GALA is also a buffered electrolyte solution containing glucose, to supplement cells with a source of cellular source of energy. The current study is the first randomized controlled trial directly comparing endothelial dysfunction caused by GALA vs saline where each patient acted as its own control. Our results are essentially consistent with the findings from the analogous study conducted by Wise et al 2016 that evaluated endothelial functions of HSVGs in various preservation solutions (including GALA and saline) through vascular reactivity. They observed that endothelium –independent smooth muscle relaxations were significantly impeded in HSVs stored in normal saline in comparison to GALA-treated HSV rings due to acute cellular injury

[197]. More importantly, HSVs stored in GALA showed statistically significant superior endothelium-dependent relaxations induced by the cholinomimetic agonist of endothelial NO production (CCh) upon comparison with HSVs stored in normal saline. In fact, the response to carbachol reflects both the responses of smooth muscle and endothelial nitric oxide release that were possibly enhanced due the presence of the eNOS substrate, L-arginine[197] These results were confirmed in the present study since saline was associated with an impairment in both endothelial-dependent and endothelium-independent relaxation. This later may be the consequence of an alteration is endogenous relaxing factors caused by a decreased action on underlying vascular smooth muscle cells subsequent to a scavenging by reactive oxygen species due to the absence of both L-arginine and antioxidants in saline. It remains debatable whether the use of antioxidants prevent saphenous vein graft dysfunction and improve patency [220], but antioxidant additives (glutathione or ascorbic acid) are believed to mitigate tissue damage caused by free radical generation causing tissue injury inflicted during preparation [170]. Unlike Wise et al, the current study showed that HSV rings contractile response to potassium chloride and phenylephrine was not significantly altered during storage in saline [197]. Nevertheless, vascular reactivity studies along studies of cellular viability and assessment of endothelial structural integrity strongly indicate that preservation in a buffered electrolyte solution with a physiologic pH is key to maintain functional and structural integrity of HSV grafts during storage.

6.1-Study limitations and future directions

Some limitations have been identified in this study. Since each patient acts as its own control, it is challenging to have enough functional rings to expose to all agonists. Even if endothelial functions are preserved in GALA, its mechanism of action remains unsolved. Investigating eNOS expression and activity, cGMP and reactive oxygen species measurement could help elucidate the way GALA preserves endothelial integrity. Continuous oxygen and temperature modulating from the moment of SVGs harvesting throughout the storage time should be investigated to determine if it would contribute to further endothelial functions preservation. Plasmalyte-A is another promising preservation solution that was reported to protect endothelial integrity and should be included in future experiments to compare with GALA and heparinized

saline. Sub-population groups of patients with various pathologies such as diabetes and acute kidney failure should be explored since they are more prone to VGF.

Chapter 7 – Conclusion

In conclusion, there is a unique window at the time of intraoperative storage of HSV during CABG surgery during which optimal *ex vivo* storage of the graft can minimize acute injury to the endothelial layer and the underlying smooth muscle. The use of normal saline as a preservation impairs endothelial functions and decreases cellular viability of the graft and is not recommended. The authors have demonstrated that a specifically designed storage graft solution such as GALA can mitigate acute injury, maintain smooth muscle function, as well as preserve the functionality of the endothelial monolayer. Though the optimal HSV graft preservation solution remains unknown, it is clear that a balanced buffered salt solution, with potentially endothelial protective additives such as arginine, antioxidants, and cellular nutrients mitigate acute injury. Overall, the results of this study support and are in concordance with data from the vast majority of fundamental and clinical studies, which suggest that GALA reduces endothelial damage and intimal hyperplasia, as well as graft remodeling and eventually decrease clinical event rates associated with venous graft failure.

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David Khalaf,[1] Marcus Krüger,[2] Markus Wehland,[2] Manfred Infanger,[2] and Daniela Grimm[1,2,3,*]

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