Effect of nitric oxide overexpressing endothelial progenitor cells on coronary artery smooth muscle cells

Sergio Guber, Division of Experimental Medicine, McGill University, Montreal

May 2013

A thesis submitted to McGill in partial fulfilments of the requirements of the degree of Master of Science in Experimental Medicine

© Sergio Guber 2013
# Table of contents

Abstract..................................................................................................................1

Résumé..................................................................................................................4

Acknowledgements................................................................................................7

Abbreviations........................................................................................................9

Introduction..........................................................................................................19

  Physicochemical properties of nitric oxide.......................................................20

  Environmental sources of NO..........................................................................22

  Nitric oxide in plants.........................................................................................24

  Medicinal use of nitrates..................................................................................25

  Nitric oxide synthase isoforms.........................................................................30

  Nitric oxide synthesis.......................................................................................31

  Nitric oxide synthase regulation.......................................................................33

  Biological functions of nitric oxide....................................................................39

  NO dysregulation.............................................................................................45
Results......................................................................................................................................77

Effect of an NO donor on SMC proliferation and migration.......................................................78

eNOS overexpression is associated with increased NO production...........................................79

eNOS overexpression increases EPC survival.............................................................................79

eNOS overexpressing hEPCs decrease hCASMC migration and proliferation..............................81

Estrogen induces NO production in hEPCs...............................................................................83

Estrogen increases hEPC survival..............................................................................................84

E₂ stimulated hEPCs decrease HCASM migration but not proliferation.................................85

eNOS and NO production in E₂-stimulated eNOS overexpressing hEPCs.................................86

Apoptosis levels on E₂ stimulated eNOS transfected hEPCs.....................................................87

Effect of eNOS transfected E₂ stimulated hEPCs on hCASMC migration.................................88

Effect of eNOS transfected E₂ stimulated hEPC on PDGF stimulated hCASMC protein kinase pathways...........................................................................................................89

Statistical Analysis...................................................................................................................90
Abstract

Arterial restenosis, which occurs in up to 20% of angioplasty patients, is characterized by an excessive vascular smooth muscle cell proliferation resulting from the removal of the endothelial cell lining. Circulating endothelial progenitor cells (EPCs) have the ability to re-colonize and repair the damaged vascular endothelium, reducing restenosis. Nitric oxide (NO) contributes to mobilization and functional activity of EPCs, and estrogen has been shown to increase circulating EPC levels and accelerate the reendothelialization process by EPCs in mice. Moreover NO is important for transducing estrogen-dependent signalling and reendothelialization. We hypothesized that overexpressing endothelial nitric oxide synthase (eNOS), alone or in combination with estrogen treatment, would potentiate the beneficial effects of EPCs in the context of restenosis.

We found that native human early outgrowth EPCs (hEPCs) did not have any effect on human coronary artery smooth muscle cell (hCASMC) proliferation and migration \textit{In vitro}, evaluated by BrdU incorporation and wound scratch assay.
respectively. In contrast, the NO donor SNAP significantly decreased the proliferation and migration of hCASMC. Thereafter, hEPCs were either transfected with a human eNOS plasmid or stimulated with 17β-estradiol (E₂) prior to being co-cultured with hCASMC. Total eNOS protein and eNOS phosphorylation levels were increased by 3- to 3.5-fold in eNOS-transfected or E₂-stimulated hEPCs, evaluated by western blot. This was associated with a 3-fold increase in NO production, performed by DAF-FM diacetate immunofluorescence (p<0.05). In eNOS-overexpressing hEPCs, enhanced Bcl-2/Bax ratio and reduced Annexin V/propidium iodide labeling indicated increased survival. Interestingly, we observed a significant (p<0.05) decrease in hCASMC migration when co-cultured with eNOS-overexpressing hEPCs, by 23%, or with E₂-stimulated hEPCs, by 56%. However, hCASMC proliferation was not affected by either eNOS-overexpressing or E₂-stimulated hEPCs.

These results suggest that overexpressing eNOS in hEPCs increases their survival and enhances their capacity to modulate hCASMC migration through
paracrine effects.
Résumé

La resténose artérielle, qui se produit dans presque 20% des patients ayant subi une angioplastie, est caractérisée par une prolifération excessive des cellules musculaires lisses vasculaires résultant de la disparition du revêtement des cellules endothéliales. Les progéniteurs endothéliaux circulants (EPC) ont la capacité de recoloniser et réparer l'endothélium vasculaire endommagé, ce qui réduit la resténose. L'oxyde nitrique (NO) contribue à la mobilisation et l'activité fonctionnelle des EPCs, et l'oestrogène a été montré pour augmenter le taux circulant des EPCs et d'accélérer le processus de réendothélialisation par les EPCs chez la souris. En outre, le NO est important pour la transduction de la signalisation oestrogène-dépendante et la réendothélialisation. Nous avons proposé que la surexpression d'oxyde nitrique synthase endothéliale (eNOS), seule ou en combinaison avec un traitement à l'oestrogène, potentialiserait les effets bénéfiques des EPCs dans le cadre de la resténose.
Nous avons constaté que les EPC humains natifs (hEPCs) n'ont pas d'effet sur la prolifération et la migration in vitro des cellules musculaires lisses de l'artère coronaire humaine (hCASMC), évaluées par l'incorporation de BrdU et le l'essai « wound-scratch » respectivement. En revanche, le donneur de NO SNAP a diminué de façon significative la prolifération et la migration des hCASMC. Par la suite, des hEPCs ont été soit transfectés avec un plasmide eNOS humain ou stimulés par du 17β-estradiol (E₂) avant d'être co-cultivés avec des hCASMC. Les niveaux d'eNOS totale et phosphorylée évalués par western blot ont été augmentés de 3 - à 3,5 fois dans les hEPCs transfectés avec eNOS ou stimulés avec E₂. Cela a été associé à une augmentation de 3 fois de la production de NO, mesuré par immunofluorescence avec DAF-FM diacétate (p <0,05). Des hEPCs surexprimant eNOS ont montré une augmentation du rapport Bcl-2/Bax et le marquage d'annexine V/iodure de propidium a été réduit indiquant une augmentation de la survie. Fait intéressant, nous avons observé une diminution significative (p <0,05) de la migration des hCASMC de 23% pendant la co-culture.
avec des hEPCs surexprimant eNOS, et de 56% avec des hEPCs stimulés avec E₂. Cependant, la prolifération des hCASMC n'a été affectée ni par des hEPCs surexprimant eNOS, ni par les hEPCs stimulés avec E₂.

Ces résultats suggèrent que la surexpression de eNOS dans des EPCs humains augmente leur survie et améliore leur capacité à moduler la migration des hCASMC via des effets paracrines.
Acknowledgements

I would like to thank first and foremost my supervisor Stéphanie Lehoux for giving me the opportunity to be part of her team. Her insight not only on scientific matters but on everyday lab life helped me learn to think “outside the box” to find novel solutions to particularly stubborn problems.

I would also like to thank the members of the lab for their support. They were always ready to give their advice, share the occasional reagent and lend a helping hand/arm. Their camaraderie made daily chores pleasant and easy going. A special mention goes for Talin Ebrahimian without whom this work would be but a fraction of what it is. Her collaboration in experiment planning and result analysis proved to be both enriching and constructive.

I would also like to acknowledge Dr. Nicoletta Eliopoulos who provided us with the eNOS plasmid created by Dr. David Courtman lab. Her input concerning mononuclear isolation and cell morphology aided us to better understand EPC physiology.
I’m also grateful for the assistance of the McGill’s Department of Experimental Medicine. They were always helpful beyond their duty and despite their work load.

Last but not least, I would like to thank the Hematology Department of the Jewish General Hospital and their nurses for their cooperation. A special mention must go for Joanne Rodriguez who I pestered on a weekly basis. Their expertise with the needle and good predisposition always made for an enjoyable moment.
**Abbreviations**

ADP: adenosine diphosphate

AIF: apoptosis inducing factor

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ATP: adenosine triphosphate

AV: annexin V

Bax: Bcl-2 associated X protein

Bcl-2: B-cell lymphoma 2

BH₄: tetrahydrobiopterin

BK: Bradykinin

BrdU: bromodeoxyuridine
BSA: bovine serum albumin

Ca$^{2+}$: calcium

CAD: caspase activated DNase

Caspase: cysteine aspartic protease

cGMP: cyclic GMP

c-Src: cellular sarcoma

Cyto C: cytochrome C

Da: Dalton

DAG: diacylglycerol

DAF-FM: 4-amino-5-methylamino-2',7'-difluorofluorescein

DAPI: 4',6-diamidino-2-phenylindole

DES: drug eluting stents
DNA: deoxyribonucleic acid

E₂: 17 β-estradiol

EBM-2: endothelial cell basal media 2

ECL: enhanced chemiluminescence

EDRF: endothelial derived relaxing factor

EDTA: ethylenediamine tetraacetic acid

EGM-2: endothelial cell growth media 2

eNOS: endothelial nitric oxide synthase

EPC: endothelial progenitor cell

EPO: erythropoietin

ER-α: estrogen receptor alpha

ERK extracellular signal-regulated kinase
FAD: flavin adenine dinucleotide

FAK: focal adhesion kinase

FcR: Fc receptor

FITC: fluorescein isothiocyanate

FMN: flavin adenine mononucleotide

GAB1: GRB2-associated-binding protein 1

G-CSF: granulocyte colony stimulating factor

GM-CSF: granulocyte/macrophage colony stimulating factor

GMP: guanosine monophosphate

GPCR: G protein-coupled receptor

hCASMC: human coronary artery smooth muscle cell

hEPC: human endothelial progenitor cell
HEPES: 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid

HIF-1α: hypoxia inducible factor 1α

HNO₂: nitrous acid

HRP: horseradish peroxidase

ICAM-1: intracellular adhesion molecule 1

IgG: immunoglobulin G

iNOS: inducible nitric oxide synthase

IP₃: inositol triphosphate

JAK: Janus kinase

kDa: kilo Dalton

L-NAA: N-amino-L-arginine

L-NAME: N-nitro-L-arginine methyl ester
L-NIO: amidin-Niminoethyl-L-ornitine

L-NMMA: N-monomethyl-L-arginine

LADMA: NN-dimethyl-L-arginine

LDL: circulating low-density lipoproteins

LOCA: N-omega-cyclosporin-L-arginine

LPS: lipopolisaccharide

M-CSF: macrophage-colony stimulating factor

MAP: mitogen-activated protein

MCP-1: monocyte chemotactic proteins 1

MMP: matrix metalloprotease

MNC: mononuclear cell

mRNA: messenger ribonucleic acid
mtALDH: mitochondrial aldehyde dehydrogenase

NaCl: Sodium chloride

NADPH: nicotinamide adenine dinucleotide phosphate

NaF: Sodium fluoride

NANC: non-adrenergic non-cholinergic

Na$_3$VO$_4$: sodium orthovanadate

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NO$_2$: nitrogen dioxide

NO$_2^-$: nitrite

NO$_3^-$: nitrate
NOS: nitric oxide synthase

\( O_2^- \): oxide anion

\( O_2^- \): superoxide

\( OH^- \): hydroxyl

\( ONOO^- \): peroynitrite

PAR-1: protease activated receptor 1

PBS: phosphate buffered saline

PARP: poly ADP-ribose polymerase

PDGF: platelet derived growth factor

PFA: paraformaldehyde

PI: propidium iodide

\( PIP_2 \): phosphatidyl inositol diphosphate
PK-C: protein kinase C

PLA2: phospholipases A

PLCβ: phospholipases Cβ

PMSF: phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride

ppm: parts per million

PVDF: Polyvinylidene fluoride

RCF: relative centrifugal force

ROS: reactive oxygen species

SH2: Src homology 2

SNAP: s-nitroso-N-acetylpenicillamine

Src: sarcoma

Ser: serine
SMoA: alpha-smooth muscle actin

SMC: smooth muscle cell

SDF-1: stromal cell-derived factor 1

STAT: signal transductor activating transcription

TBS-T: tris-buffered saline and Tween 20

TGF-β: transforming growth factor beta

Thr: threonine

TLR4: Toll-like receptor-4

TMB: 3,3’,5,5’-Tetramethylbenzidine

VCAM-1: vascular cell adhesion molecule 1

SMC: vascular smooth muscle cell

VEGF: vascular endothelial growth factor
Introduction

Alfred Nobel (1833-1896), the Swedish chemist engineer and entrepreneur, is best known for the annual international awards that bear his name. After an accident with nitroglycerin in one of his factories that took the life of his brother Emil and four other workers, he decided to find a way to make nitroglycerin safer to handle, and eventually patented dynamite in 1867 [1].

During most of his life, Alfred Nobel had a precarious health. He complained of various ailments ranging from indigestions to depression. In the course of his last years, he suffered from angina pectoris (intense chest pain), so it wasn’t without a sense of irony that his physicians suggested nitroglycerin as a treatment for his heart condition, yet Nobel refused to take it [2]. Despite its unknown physiological mechanism of action, nitroglycerin was prescribed to heart patients since 1879. It took more than 100 years to determine that it was through the biosynthesis of nitric oxide (NO) by mitochondrial aldehyde dehydrogenase (mtALDH) that nitroglycerin exerted its effects [3]. Nitric oxide induces the relaxation of the
smooth muscle cells in the vasculature producing an enlargement of the blood vessels and therefore increasing the blood flow. As a consequence, more oxygen is delivered to the heart muscle and the pain is reduced [4].

**Physicochemical properties of nitric oxide**

Nitric oxide was discovered by the English chemist Joseph Priestley (1733–1804) in 1772, who named it “nitrous air”. Priestley is also responsible for the discovery of other “airs”, including oxygen, which he named "dephlogisticated air" [5]. Nitric oxide, also known as nitrogen monoxide, is a 30 Da binary molecule where the nitrogen and oxygen atoms are linked by a double bond. As a result of the similar electronegativity of nitrogen and oxygen, the molecule possesses a small dipole moment making it mainly hydrophobic and allowing it to easily diffuse through cell membranes [6]. Due to its short half life of 2-5 seconds, NO serves as an autocrine or paracrine messenger molecule for many physiological functions [7]. Moncada et al. have reported that NO is converted to nitrite and nitrate in the blood within 10 seconds of formation [8]. It has been estimated that if biologically
active nitric oxide levels are 0.001 μM, important NO concentrations could be
found in a sphere of 340 μm [9].

The molecule is considered a free radical since it has unpaired electrons in the
valence shells of its atoms [10]. Nevertheless, it is relatively stable, reacting
mainly with molecules with unpaired electrons which are usually other free
radicals (O$_2^-$ superoxide ion, OH- hydroxyl ion) or transition metals, such as heme
iron containing molecules (hemoglobin, cytochromes, peroxidases, and others)
[11]. *In vitro*, NO degrades mainly into NO$_2^-$ (nitrite) while *in vivo*, it predominantly
produces NO$_3^-$ (nitrate) [12].

With a melting point of -164° C and a boiling point of -152° C, at room
temperature, nitric oxide is a colorless, extremely toxic oxidizing gas that causes
minor irritation of the eyes and respiratory tract at concentrations of 25 ppm and
can be lethal at 200-700 ppm, even after short exposures. Continued exposures
to low concentrations can result in pulmonary oedema, headache, and dental
erosion among other consequences [13]. In the blood, NO oxidizes hemoglobin
to methemoglobin. Since methemoglobin is unable to carry oxygen, this can lead to death by hypoxia if methemoglobin levels reach 70% [14].

**Environmental sources of NO**

In nature, nitrogen oxides can be generated by lightning, bushfires and microbial processes. Already in 1857, the German chemist Justus von Liebig suggested that lightning could fix atmospheric nitrogen that would eventually be washed by rainwater into the earth [15]. Nowadays, this is believed to be the main source of nitric oxide in the troposphere [16]. The high temperature in the vicinity of the lightning bolt causes oxygen and nitrogen to react forming NO [17].

Another source of NO is the metabolism of soil bacteria called rhizobia that live in symbiosis with plant roots and have the ability to switch from O₂ respiration to using nitrates when faced with an oxygen shortage. These bacteria can produce nitric oxide through a pathway that involves an NO synthase-like enzyme [18].

Human sources of nitrogen oxides come as a result of burning fossil fuels, such as oil and coal. Much like in the case of lightning, high temperatures catalyze the
reaction of nitrogen and oxygen to produce nitric oxide [19]. During the 1970s, nitric oxide created as a by-product of different human activities was singled out as an air pollutant involved in the formation of acid rain [20], ozone depletion [21] and greenhouse effect [22].

During acid rain formation, nitric oxide reacts with oxygen producing nitrogen dioxide (NO$_2$), which can be broken down by sunlight to produce more NO and an oxygen radical (O$^{2-}$). When the oxygen radical reacts with water, it gives the hydroxyl radical (OH-) which can react with nitric oxide to produce nitrous acid (HNO$_2$) [20].

During the 1950s, evidence was presented showing that NO could react with ozone to produce nitrogen dioxide and oxygen, reducing the overall ozone level [21]. Also, nitrous oxide is stable enough to reach the stratosphere, where it can be converted into NO and further deplete ozone [23].

Nitric oxide has also been reported to combine with ozone and water to produce photochemical smog [24] and it has been shown to have 298 times more impact
per unit weight (global warming potential) than carbon dioxide. Therefore, despite its low concentration, NO is the fourth contributor to greenhouse gases behind water vapour, carbon dioxide and methane [22].

**Nitric oxide in plants**

In plants, nitric oxide functions as a signalling molecule, acts against oxidative stress and also takes part in plant-pathogen interactions. It can be produced by enzymatic and non-enzymatic pathways. Enzymatic pathways involve cytosolic nitrate reductase, NO synthase or NOS-like enzymes, and nitrite:NO reductase while the non-enzymatic pathway consists of nitrite dismutation and nitrate at acidic pH values [25].

Nitric oxide has been characterized as a phytohormone and as a plant growth regulator [26]. At lower concentrations (5µM), NO was seen to increase leaf expansion rate, whereas no supplemental effect was seen at higher levels (12µM) [27], and in some plants it even inhibited leaf growth [28]. While in some
species nitric oxide delayed apoptosis, in others, high concentrations of nitric oxide induced reactive oxygen species-dependent cell death [29].

Mitochondrial respiration has been shown to be reduced in plants exposed to NO, which was accompanied by an increase in cell death [30].

Also, while NO was reported to increase chlorophyll content in maize leaves [31], it decreased the net photosynthetic rate in other plants [32]. This decline can be explained by the reduction in the levels of the proteins that regulate photosynthesis [33].

**Medicinal use of nitrates**

Amyl nitrite was first synthesized by the French chemist Antoine Balard in 1844 [34]. Further investigations by the English chemist Frederick Guthrie observed that inspiration of the vapours of a paper moistened with two drops of amyl nitrite produced within the minute a throbbing of the arteries in the neck followed by a flushing of the neck, temples and forehead, and an acceleration of the heart [35]. Therefore, he proposed the compound as a resuscitative for drowning and
fainting, not realising that in fact it caused a drop in blood pressure, as the veterinarian Arthur Gamgee later determined [36].

Working with Dr. Gamgee’s, the Scottish M.D. Lauder Brunton was the first physician who used amyl nitrite to treat angina pectoris. The patient suffered recurring chest pain usually from 2:00 to 4:00 h a.m. and during the attacks his pulse greatly increased. Although he thought that the symptoms were caused by hypertension [36], it was only in 1903, that the physiologist François-Franck suggested that amyl nitrite acted as a coronary vasodilator [37].

On a similar note, when the Italian chemist Ascanio Sobrero tasted nitroglycerin, a compound of his invention, he noticed that it was sweet, pungent and aromatic but “great precaution should be used, for a very minute quantity put upon the tongue produces a violent headache for several hours” [38].

In 1878, the British doctor William Murrell, familiar with Dr Brunton’s work on amyl nitrite, used nitroglycerin to treat angina. He noticed that, while amyl nitrite acted for only a few seconds, nitroglycerin provided almost an hour of relief [39].
Although unknown at that time, this is due to the fact that nitroglycerin cannot readily pass the plasma membrane therefore delaying the production of its by-product nitric oxide in the mitochondria [40].

However, chronic exposure to nitrogen containing compounds can lead to tolerance as seen on workers in the explosive industry. This phenomenon is due to oxidation of sulphydryl groups in vascular smooth muscle cells which prevents the stimulation of guanylate cyclase, a fundamental step in vasodilation [41].

Nevertheless, this tolerance is reversible and the workers who weren’t exposed during the weekend experienced the same nitroglycerin-associated discomfort on their return to work [42]. To prevent this, they rubbed their skin with a piece of cloth moistened with nitroglycerin or even their work clothes impregnated with the compound at home [43].

But the worst side effect of their chronic exposure was the development of dependence to organic nitrates. The withdrawal during the weekends caused
overcompensation and arterial spasm which led to non-exertional cardiac pain and even sudden death [44].

In the 1970s, the pharmacologist Ferid Murad showed that nitrate-containing compounds stimulated guanylate cyclase causing an increase in cyclic GMP (cGMP) levels in tracheal smooth muscle and resulting in vascular relaxation. He suggested that cGMP activation was due to nitric oxide formation since the gas also increased guanylate cyclase activity [45].

Working independently, pharmacologist Robert Furchgott and his colleague, John Zawadzki, noted that acetylcholine, a strong vasodilator, didn’t produce relaxation of blood vessel preparations in vitro, whereas others were very responsive [46]. After further investigation, it was discovered that this discrepancy was the result of “unintentional rubbing of the vessel intimal surface during preparation”, thus establishing the link between endothelial cells and arterial smooth muscle relaxation [47].
Later, Furchgott et al. recognized that guanylate cyclase could be activated by nitric oxide [48] in coincidence with the work of Murad showing that nitrite-containing vasodilators operated via the release or generation of NO [49]. Furchgott’s group proposed that arterial relaxation due to bradykinin or histamine was the result of the same substance which they named endothelial derived relaxing factor (EDRF) [50]. The group lead by the pharmacologist Louis Ignarro finally identified EDRF as nitric oxide and the two groups eventually agreed that they were one and the same [51].

Ignarro showed that EDRF and NO activated soluble guanylate cyclase by the same mechanism, that pyrogallol inhibited cGMP accumulation by both EDRF and NO, and finally, that superoxide dismutase enhanced cGMP accumulation and arterial relaxation caused by EDRF and NO [52].

A few months before, Salvador Moncada had published a paper in Nature suggesting that EDRF and NO were identical, using similar techniques as Ignarro et al. [53]. On 1998, the Nobel in Physiology or Medicine was awarded to
Furchgott, Ignarro and Murad “(...) for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system” [54]. Moncada was ignored although he presented findings similar to Ignarro’s six months earlier.

**Nitric oxide synthase isoforms**

Nitric oxide is formed from L-arginine, NADPH and oxygen during a series of oxidation reactions catalyzed by a nitric oxide synthase (NOS) enzyme. Unlike synthetase enzymes, synthases don’t need adenosine triphosphate (ATP) for their action [55].

Three different NOS enzymes have been identified. The two constitutive isoforms, neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), depend on calcium/calmodulin levels for their activation. On the other hand, the inducible form (iNOS or NOS2) is calcium/calmodulin independent and, unlike the constitutive counterpart, can be downregulated by glucocorticoids. All three variants are products of three different genes located in chromosomes 12 (eNOS), 7 (nNOS), and 17 (iNOS; also known as macrophage NOS) [56].
The subcellular localization and regulation of their activities are determined by their role in the organism. While eNOS is located on the cell membrane, nNOS and iNOS are found in the cytosol [57].

Endothelial NOS can be found mainly in thrombocytes and endothelial cells.

Neuronal NOS is also found in thrombocytes but also in neurons, β-cells of pancreas, muscle, lung, stomach, uterine epithelial cells, and in endothelial cells of afferent and efferent arterioles [57-61]. Finally, the inducible NOS form is mainly involved in immunological responses and is more abundant in macrophages, neutrophils, Kupffer cells, macro- and microglia [57, 62].

**Nitric oxide synthesis**

NOS is a complex enzyme both in structure and regulation. The active form consists of a homodimer where each 130-150 kDa subunit contains a calmodulin-binding domain, a reductase domain, and an oxygenase domain [63]. The reductase domain uses flavin adenine dinucleotide (FAD) and flavin adenine mononucleotide (FMN) as cofactors. It is believed that FAD is the primary
NO (nitric oxide) synthesis by an eNOS (endothelial nitric oxide synthase) homodimer begins with the binding of Ca^{2+}/CaM (calcium/calmodulin) complex to the CaM-binding domain which facilitates the e\textsuperscript{-} (electron) flow from NADPH (nicotinamide adenine dinucleotide phosphate) to the FAD (flavin adenine dinucleotide) and FMN (flavin adenine mononucleotide) cofactors. The BH4 (tetrahydrobiopterin) cofactor catalyzes the oxidation of L-Arg (L-Arginine) by the heme group producing NO and L-Citr (L-citrulline). eNOS can be regulated by both activating (Serine 1177) and inhibiting (Threonine 495) phosphorylations.

Endothelial nitric oxide synthase dimer, adapted from Campen et al. (2009)
FMN then transports the electrons from FAD to the heme of the opposite subunit’s oxygenase domain, which has heme, L-arginine and tetrahydrobiopterin (BH4) binding sequences [62].

In a 2-step process, the electrons at the heme site are used to activate oxygen and oxidize L-arginine to L-citrulline and NO. In the first part, L-arginine is hydroxylated to Nω-hydroxy-L-arginine, and in the next phase, Nω-hydroxy-L-arginine is oxidized to L-citrulline and NO [64]. The binding of calmodulin facilitates the electron flow from NADPH to heme [65]. Also, NOS dimerization is required for BH4 binding [66].

**Nitric oxide synthase regulation**

All NOS enzymes have a basal level of NO production that regulate physiological processes, yet the constitutive forms (eNOS and nNOS) depend on Ca^{2+} levels for their activation, while the inducible form becomes fully activated even at low Ca^{2+} concentrations [62].
The activity of iNOS can be increased after stimulation by extracellular stimuli such as bacterial endotoxins and cytokines (interleukin-1, interleukin-2, interferon-γ, tumor necrosis factor). Lipopolysaccharides (LPS), a cell wall component of bacteria and fungi, bind to the LPS-binding protein which delivers LPS to CD14, a high affinity LPS receptor. Toll-like receptor-4 (TLR4) interacts with the CD14-LPS complex activating intracellular signalling cascades that result in the translocation of NF-κB from the cytoplasm to the nucleus where it induces iNOS gene expression [67]. Cytokines can also increase iNOS transcription by the activation of the JAK (Janus Kinase) and STAT (Signal Transducers and Activators of Transcription) pathways through the IFNR1 (interferon receptor-1) and IFNR2 complex. iNOS activity appears 6 to 8 hours after induction, which is the time it takes to activate the gene and synthesize the enzyme. Once achieved, iNOS can generate up to 1000 times more NO than the constitutive forms and may continue for days. iNOS expression is associated with pathological conditions since it generates amounts of NO that are toxic for the cell. Thus, it is
believed that iNOS is important in the suppression of bacterial and tumor cells and during the appearance of lipid peroxidation disorders [68].

The cytotoxicity of NO is due to the formation reactive nitrite and oxygen intermediates. It has been postulated that NO binds to complexes I and II of the mitochondrial electron transport chain, resulting in cell death [60].

On the other hand, NO production by eNOS and nNOS can be rapidly activated by an increase in cytoplasmic levels of Ca^{2+} in the presence of calmodulin [69].

NOS can also be activated through pathways that involve the influx of Ca^{2+} through ion channels activated by ionotropic mediators. This further induces the mobilization of Ca^{2+} from the endoplasmic reticulum. Then, a Ca^{2+}/calmodulin complex is created which activates NOS. Another pathway involves metabotropic receptors, a type of G protein-coupled receptors, which leads to the activation of membranous NOS forms, adenylate cyclase, and phospholipases A and Cβ (PLA2 and PLCβ) [70]. Phosphatidyl inositol diphosphate (PIP2), located in the inner membrane, is converted to inositol triphosphate (IP3) and diacylglycerol
(DAG) by PLCβ. IP₃ then binds to an endoplasmic receptor membrane that induces the release of Ca²⁺ from the endoplasmic reticulum to the cytosol. Finally, Ca²⁺ binds to calmodulin promoting NOS dimerization at the oxygenase domain [71].

Hemodynamic forces are thought to be the most important stimulus leading to NO production. Endothelial cells have the ability to increase NO synthesis in response to shear stress within seconds of sensing the stimulus. The viscous drag of blood over endothelial cells as well as wall vessel stretching due to the pulsatile nature of blood flow results in the activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway, protein kinase A and AMP-activated protein kinase (AMPK). All these kinases phosphorylate eNOS at Ser1177, leading to increased eNOS activity [72, 73].

Also, estrogens are important modulators of eNOS-derived NO production. In particular, 17β-estradiol (E₂) binds to the estrogen receptor (ER)-α, which leads to eNOS activation by phosphorylation of serines 615 and 1177 through the c-
Scr/PI3K/Akt pathway [74]. Additionally, it has been shown that E2 metabolites are also able to activate eNOS via an AMPK dependent pathway [75]. eNOS activation by estrogens has been shown to enhance a long term increase both in the formation of NO as well as in the expression of eNOS mRNA [76].

Even though the mechanism involving the signal transduction of shear stress is not fully understood, it is known to be mediated by several signalling molecules including adaptor protein Gab1, tyrosine phosphatase SH2, and Src kinase activation of VEGF receptor 2 [77, 78]. Scr kinase mediated NO upregulation has also been reported to occur by a short term increase in eNOS transcription and long term eNOS mRNA stabilization [79].

It has been shown that bradykinin, acetylcholine or shear stress mediated stimulation of endothelial cells results in calcium influx leading to the activation of eNOS. The synthesized nitric oxide diffuses to the adjacent smooth muscle cells causing cGMP mediated relaxation by two mechanisms. First, cGMP provokes a decrease in calcium levels and increasing the permeability of potassium channels
Second, cGMP activates kinases that phosphorylate the myosin light chain, preventing myosin-actin interaction [81, 82]. The binding of nitric oxide to the heme site of guanylate cyclase enhances cyclic GMP production inhibits calcium entry into the cell leading to smooth muscle cell relaxation [80].

There are several ways by which nitric oxide synthesis can be downregulated. Among the most relevant is the inhibition by L-arginine derivatives such as by N-monomethyl-L-arginine (L-NMMA), NN-dimethyl-L-arginine (LADMA) and N-nitro-L-arginine methyl ester (L-NAME). They compete with L-arginine inhibiting all NOS isoforms. Other inhibitory substances are specific to iNOS, like L-canavanine, N-amino-L-arginine (L-NAA), amidin-Niminoethyl-L-ornitine (L-NIO), amino guanidine) and eNOS N-omega-cyclosporin-L-arginine (LOCA) [83].

NO synthesis can also be blunted substances such as diphenyleneiodonium, which inhibit enzymes that use NADPH. NOS activity can be decreased with calmodulin antagonists (trifluoperazine, chlorpromazine, calmidazolium ) by competitive inhibition of calmodulin receptors. Synthesis inhibition of BH₄ with
methotrexate and N-acetyl-5-hydroxytryptamine leads to NOS downregulation.

Finally, NO can react with the heme group inhibiting NOS by negative feedback [56].

**Biological functions of nitric oxide**

Although the present project will focus mainly on the role in the vasculature, nitric oxide plays a myriad of physiological functions ranging from bronchodilation and modulation of the hair cycle, to neurotransmission and immune response [84-87]. NO fulfills all the requirements to be considered a non-adrenergic non-cholinergic (NANC) neurotransmitter: it is synthesized in neurons, it can stimulate NANC neurons, and it has a mechanism for inactivation (it rapidly breaks down) [88, 89]. The increase in neuronal cGMP is associated with changes in electrogenesis that are believed to participate in long term synaptic potentiation, which is the basis of learning and memory [90].
Nitric oxide production can be enhanced by many circulating substances like catecholamines, neurohypophyseal hormones, sex hormones, autacoids and growth factors.

Bradykinin (BK) is an autacoid that has the ability to induce endothelium-dependent relaxation, mainly through the release of NO. BK acts on GPCRs on the endothelial cell membranes and activate eNOS by calcium-dependent and independent mechanism (phosphorylation of Ser^{177} and dephosphorylation of Thr^{495}) [91]. Additionally, BK can transactivate the vascular endothelial factor (VEGF) receptor promoting NO formation [92].

The stimulation of endothelial cells by vasopressin and oxytocin increases NO formation and produces arterial relaxation [93].

Growth factors like VEGF, basic fibroblast growth factor, platelet-derived growth factor, and insulin are able to modulate eNOS activity via phosphorylation and calcium dependent mechanisms [94].
During blood coagulation events, the serine protease thrombin transforms fibrinogen to fibrin helping stabilize the clot. Mediated by the protease activated receptor (PAR) -1, thrombin immediately increases NO production by up to 20 times. This is achieved by an increase in calcium levels, which lead to a Ca²⁺/calmodulin dependent eNOS activation [95]. Thrombin also enhances eNOS activity by phosphorylation of Ser¹¹⁷⁷ via an AMP-activated protein kinase pathway [96]. Factor Xa, a protease involved in the coagulation cascade, has been reported to increase NO formation independently of PAR-1 [97]. Platelet aggregation and activation has been seen to increase calcium concentration resulting in the release of vasoactive factors like serotonin and ADP. These factors act on GPCRs located on the membrane of endothelial cells inducing a burst of NO after Ca²⁺/calmodulin eNOS activation [98]. These clotting related mechanisms help dilate the blood vessel and prevent ischemia in case of a thrombotic incident.
Nitric oxide also has the ability to either block or initiate apoptosis depending on the presence or absence of stimulatory cosignals [99]. Apoptosis, or programmed cell death, involves a cascade of events that results in DNA cleavage and phagocytosis of the resulting apoptotic bodies by neighbouring cells in response to certain stressors. Unlike necrosis, macrophages are able to engulf the dead cell fragments before cell rupture occurs thus preventing the onset of an inflammatory response [100]. Nitric oxide-damaged DNA has been shown to induce a stress response in mammalian cells through a poly ADP-ribose polymerase (PARP) dependent pathway that leads to cell death due to ATP depletion [101].

Caspases (cysteine aspartic proteases) are a family of cysteine proteases that help propagate the apoptotic signal, eventually activating caspase-3 or 7 which is considered as the point of no return in the cell destruction process [102]. Poly ADP-ribose polymerase, which is cleaved during NO-mediated apoptosis, is an
established target of caspase-3 [103]. Also, the inhibition of NOS has been shown to block cytokine-induced caspase activation [99].

The B-cell lymphoma-2 (Bcl-2) gene encodes for a family of apoptosis regulator proteins and has been hallmarked as “the prototypic regulator of mammalian cell death” [104]. Constitutive expression of Bcl-2 protein by transfection has been reported to protect cells from apoptosis in a wide variety of conditions [105]. NO-mediated apoptosis been associated with decreased levels of Bcl-2 [106] and the upregulation of the proapoptotic Bcl-2 associated protein Bax [103]. Bcl-2 has acts as a signal terminator in NO-mediated apoptosis and also interferes with the activation of caspase-3.

On the other hand, administration of various NO donors attenuated the increase of Bax expression, caspase activation and DNA fragmentation. Apparently, the relative abundance of the superoxide anion and NO redirect the apoptotic signals towards cell survival upregulating protective genes [107].
Nitric oxide and apoptosis

Increased intracellular Ca\textsuperscript{2+} (calcium) levels lead to eNOS (endothelial nitric oxide synthase) activation and NO (nitric oxide) production. Mitochondrial O\textsubscript{2}\textsuperscript{-} (superoxide) may react with NO to produce ONOO\textsuperscript{-} (peroxynitrite) causing DNA damage and PARP (poly ADP-ribose polymerase) activation. PARP can deplete ATP and stimulate the release of mitochondrial AIF (apoptosis-inducing factor) resulting in DNA fragmentation. Also, Bax (Bcl-2 associated X protein) upregulation by stress-activated p53 induces the release of mitochondrial Cyto C (cytochrome C) leading to the activation of caspases (Casp). Casp-3 cleaves the inhibitor of CAD (caspase activated DNase) freeing CAD to cleave DNA. Bcl-2 (B-cell lymphoma 2) inhibits Bax.
Furthermore, nitric oxide has protective effects during ischemia-reperfusion, myocardial injury and reactive oxygen species cytotoxicity [108]. Some propose that the antiapoptotic role of NO is associated to NF-κB activation [109].

Low dose NO induces autoprotection in hepatocytes by decreasing the activity of caspase-3 through direct interaction [110].

In summary, depending on relative abundance of different chemical species, NO can induce apoptosis by caspase activation, chromatin condensation and DNA fragmentation, or it can induce cell survival by radicals interactions, caspase inhibition and protective genes upregulation.

**NO dysregulation**

An appropriate level of nitric oxide availability is important for a normal physiology. Both excess and shortage of NO have been associated with pathological conditions. For example, whereas decreased levels of NO have been implicated with the development of hypertension [111], elevated levels of NO have been reported to cause cytotoxicity [111].
Pathologically elevated amounts of NO can result in the nitrosylation of the cytochrome heme group thus blocking the electron transport of the respiratory cycle in the mitochondria [111]. It can also lead to the suppression of the activity of antioxidant enzymes (catalase, superoxide dismutase), DNA damage by deamination and glycolytic ATP synthesis blockage [57].

**Endothelium**

The endothelium consists of a thin layer of cells that cover the luminal surface of all blood vessels. These cells are called endothelial cells and they constitute a semipermeable barrier that regulates the exchange of circulating nutrients and factors between the blood and the underlying vessel wall. The endothelium is involved in the regulation of blood pressure, angiogenesis, blood clotting and atherosclerosis, among other roles [112, 113].

Endothelial cells can regulate blood pressure through the conversion of vasoactive factors like angiotensin converting enzyme, which catalyzes the
production of angiotensin II (a proinflammatory and vasoconstrictor peptide), or by the release of nitric oxide and prostacyclin (vasodilators) [114, 115].

The release of vascular endothelial growth factor (VEGF) is known to control endothelial cell proliferation, migration and vessel formation [116].

It is important to note that the endothelium plays an important role in the regulation of the proliferation of the underlying smooth muscle cells of the vasculature [117]. Nitric oxide release by endothelial cells has been shown to inhibit both platelet derived mitogens and vascular smooth muscle cell mitogenesis [118, 119]. NO has been shown to prevent cyclin D1 and A expression by upregulating p21, a cyclin dependent kinase 2 inhibitor [120]. Consequently, cells will be arrested at the G0/G1 cell cycle phase. Also, NO modulates extracellular matrix collagen type I and II synthesis and smooth muscle cell migration, both of which contribute to vascular remodelling [121].
Vascular smooth muscle cells

Vascular smooth muscle cells originate from different embryological sources depending on the vessel type. For example, aortic arch SMC derive from the neuroectoderm whereas those from the descending aorta derive from the mesoderm [122]. During embryogenesis, vascular development begins with the formation of primitive endothelial tubes from circulating endothelial cell progenitors that eventually recruit smooth muscle cell progenitors, the pericites [123]. As vessels mature, SMC serve as the biosynthetic, proliferative and contractile components of the vessel wall. SMC gradually become associated with the endothelium, producing and organizing extracellular matrix molecules [124].

SMC exist in two clearly differentiated states: a quiescent contractile phenotype and a synthetic migratory and proliferating phenotype. The switch between the quiescent and the synthetic phenotypes is evidenced by the expression pattern of SMC marker proteins and it is known as phenotypic switching [125]. The most
common SMC marker is SMαA (alpha-smooth muscle actin) which can make up to 40% of the protein content of the cell and 70% of the actin population. Even though SMαA expression is not sufficient criterion on its own to define a SMC precursor, it provides a mean for identification of presumptive SMC [126].

Quiescent SMC are characterized by the expression of contractile markers such as smooth muscle myosin heavy chain, smooth muscle α-actin, h-caldesmon, and calponin, which are important for the regulation of contraction [127]. Upon injury, SMC re-enter the cell cycle and show a decrease in the expression of contractile markers and increased osteopontin, MMP-1 and MMP-3 production [125].

In mature vessels, SMC reduce the secretion of ECM proteins and upregulate the production of intracellular myofilaments. The predominant SMC phenotype in mature blood vessels is the quiescent or contractile phenotype. These differentiated SMC regulate blood pressure by controlling the diameter of the blood vessel [127]. On the other hand, the synthetic phenotype appears during
the response to vessel injury. During pathogenic vascular remodeling such as atherosclerosis and restenosis, the non-contractile phenotype (de-differentiated cells), have reduced protein expression for contractile function but increased capacity to produce extracellular matrix proteins. Thus, instead of regulating contraction, the synthetic phenotype controls vascular construction [128]. The mechanism of intimal hyperplasia can be divided in an inflammatory phase, a cellular proliferation phase and an extracellular matrix remodelling phase. After the injury, mediators such as growth factors and cytokines are released by platelets and inflammatory cells promoting changes in SMC proliferation, migration and ECM production [129]. SMC retain remarkable plasticity and can dedifferentiate to a synthetic phenotype. This is extremely important because it enables the repairing of the vasculature after injury, but it can also be disadvantageous because it can lead to abnormal responses contributing to restenosis [130].
During the vascular remodelling process, SMC proliferation and migration is mediated by signalling molecules such as the adhesion molecules osteopontin and vitronectin [131]. During later stages, accumulation of new extracellular matrix also occurs leading to the thickening of the artery wall [132].

A fatty streak consists of monocytes and macrophages loaded with engulfed oxidized LDL (foam cells) and T-lymphocytes. After cycles of mononuclear cell accumulation, SMC migrate to the intima and the fatty streak is converted into a mature fibro-fatty atheroma that further enlarges the lesion [133]. Even though the accumulation of lipid-loaded macrophages is the trademark of fatty streaks, the appearance of fibrous tissue characterizes the more advanced stage of the lesion. Accumulation of migrated SMC is responsible for the production of ECM proteins [134]. Such ECM remodelling stabilizes the plaque preventing it from rupturing. However, in the context of restenosis this process leads to neointimal thickening and rigidification of the vessel, which is detrimental [135].
In the native vessel, SMC are surrounded by ECM consisting in mainly collagens type I and III, which are important in maintaining tissue structure and cell function [136]. MMPs localized in the cell surface degrade ECM promoting the change from a quiescent contractile SMC to a synthetic state capable of migrating and proliferating [137]. Of particular interest are MMP-2 and MMP-9 which are upregulated shortly after mechanical injury and are associated with SMC migration [138, 139]. Other factors capable of inducing SMC migration from the media to the intima include growth factors such as PDGF, FGF-2 and inflammatory cytokins such as interleukin (IL)-1, IL-4 and TNF-α [140]. In addition, SMC proliferation and migration is known to be inhibited by NO and transforming growth factor (TGF)-β [141]. Intracellular signalling involved in SMC migration include PI3K, protein kinase (PK)-C, FAK and MAPKs (ERK1/2) [142]. Finally, the cycles of mononuclear cell accumulation, SMC proliferation and formation of fibrous tissue result in the enlargement and restructuring of the injury.
site and the formation of a fibrous cap overlying the necrotic tissue and lipids, characteristic of the advanced lesion [143].

**Atherosclerosis**

Endothelial dysfunction is the cornerstone of atherosclerosis and is defined by a dysregulation of the endothelium leading to an increased adhesion molecule expression, cytokine release, and reactive oxygen species production [144]. Furthermore, an imbalance of vasodilating and vasoconstricting substances produced by the endothelium leads to hypertension, which further exacerbates atherosclerosis. In atherosclerosis, circulating low-density lipoproteins (LDL) permeate and accumulate beneath the endothelium. Then, LDL becomes oxidized by free radicals and this triggers an immune response that involves the expression of adhesion molecules that recruit inflammatory cells, mainly macrophages and T-lymphocytes. The oxidized LDL is engulfed by macrophages which become foam cells. Not being able to process the oxidized LDL, foam cells grow and finally undergo apoptosis on the artery wall. As a consequence, more
cells are recruited repeating the cycle which can result in plaque rupture or compromised blood flow inducing a major cardiovascular event [145]. NO exhibits potent anti-atherosclerotic properties that affect the formation and development of the disease. During the early stages, NO has been shown to inhibit LDL-induced expression of the monocyte chemotactic proteins (MCP)-1, which is involved in the recruitment of monocytes into the artery wall [146]. NO also seems to prevent the expression of endothelial cell adhesion molecules like the vascular cell adhesion molecule (VCAM)-1 and the intracellular adhesion molecule (ICAM)-1 thus affecting not only the recruitment but also the leukocyte adhesion [147, 148].

Nitric oxide may also help preventing the progression of macrophages into foam cells by inhibiting the expression of the macrophage-colony stimulating factor (M-CSF), a known regulator of macrophage growth and differentiation. NO is able to prevent the expression of these atherogenic factors partly due to the induction and stabilization of the NF-kB inhibitor. NF-κB is a transcription factor involved in the regulation of numerous genes involved in proliferative and inflammatory
responses during the pathogenesis of atherosclerosis [149]. Also, nitric oxide reduces the expression of matrix metalloproteases (MMP)-2 and -9 which are in charge of degrading collagens that can lead to plaque instability and rupture [150]. Inversely, the chronic inhibition of NO by NO synthase inhibitors has been shown to accelerate the development of atherosclerotic lesions, further supporting the protective role of NO in atherosclerosis [151].

**In-stent balloon angioplasty**

If left untreated, arterial occlusion due to atherosclerosis can become life-threatening. In order to treat the stenotic (narrowed) vessel a therapeutic approach called in-stent balloon angioplasty can be performed in order to restore proper blood flow. During the procedure, a catheter is inserted in the artery up to the point of obstruction where a balloon is inflated crushing the lipid plaque in the vessel wall and restoring blow flow. The balloon also deploys a stent that is left in the artery to prevent it from collapsing and then the catheter is removed. Unfortunately, as the balloon expands, it also destroys the endothelium
overlaying the vessel, and with it, its regulatory functions [152]. In the absence of endothelial cells, the underlying smooth muscle cells start proliferating and within 6 months, 20% of the patients suffer from restenosis due to SMC encroachment [153].

In this context it is important to find an effective way to prevent SMC proliferation. Among the different approaches that have been made in this direction, drug eluting stents (DES) have been shown to significantly decrease stenosis reoccurrence. DES are usually treated with antimitotic agents like immunosuppressant drugs that inhibit smooth muscle proliferation and as a result, reduce neointimal formation [154]. Even though DES improve patient prognosis, they also have disadvantages proper to their nature, namely that the drugs used to decrease SMC proliferation also delay re-endothelialization in the stented segment and increase the risk of thrombosis. Since inflammatory and thrombotic pathways have common signalling pathways, inflammatory responses to drug or stent could result in thrombosis. When the subendothelial matrix is
exposed following angioplasty, platelets and fibrinogen adhere to the injured site triggering a cascade that involves the expression of adhesion molecules and leukocyte recruitment. Arterial segments located distally to the stents are more prone to have impaired endothelial function due to drug accumulation in the vessel wall [155].

**Endothelial progenitor cells**

Endothelial cells usually remain quiescent as a result of contact inhibition. However, with age or after mechanical injury, cells may detach and die or undergo apoptosis, which presents a major cardiovascular risk factor. These cells can be replaced by endothelial progenitor cells (EPC) which are derived from the bone marrow into the circulation repairing the damaged endothelium. EPCs promote endothelial regeneration and neovascularization [156].

There is not a clear consensus regarding the characterization of EPCs. Previously, it was believed that CD-34 positive cells developed into EPCs but further studies showed that the CD-34 surface antigen is common to both EPC
and SMC progenitors. The commitment towards either cell lineage will depend on their milieu of development that will promote one of the phenotypes [157]. Two subpopulations have been identified: early outgrowth EPCs and late outgrowth EPCs. Early outgrowth EPCs are able to create colony forming units and differentiate into endothelial cells within 7 days and have a low proliferative capacity.

EPCs can be recruited and activated to a site of injury by the hypoxia inducible factor (HIF)-1α. As a consequence, stromal cell derived factor 1 levels and erythropoietin synthesis are augmented, which result in increased EPC numbers [158].

The most common antigens used to characterize EPCs are CD-34, CD-133, CD-45, CD-31, VEGF receptor-2, von Willebrand factor, and CD-3 (used as a negative marker). Another popular method is the labelling of cells with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine acetylated low-density lipoprotein (LDL), and endothelial-specific lectin [159]. But the ultimate assay to
determine true EPC identity is the ability to form 3 dimensional tubes on a matrigel assay [160].

The level of circulating EPCs can be influenced by many different factors. Many drugs like atorvastatin, simvastatin, mevastatin, rosiglitazone and estrogens are known to increase the count and migratory capacity of EPCs [161]. There are contradictory findings regarding various cardiovascular risk factors: while some studies affirm that smoking, hypertension and physical activity have no significant impact on EPC count, others have reported diminished basal EPC numbers in smokers or obese individuals [162, 163]. At the same time, exercise training has been shown to up-regulate the number of circulating EPCs [164].

Also, EPC levels have a good predictive value on the overall cardiovascular health. It has been reported that EPC numbers are inversely correlated to coronary artery disease risk factors [165], whereas hypertensive, diabetic or ischemic stroke patients had significantly reduced EPC levels [166].
We already mentioned that estrogens can stimulate endothelial NO production through eNOS phosphorylation in an ER-α/PI3K/AKT-dependent pathway [74]. It has been reported that estrogen-stimulated EPCs isolated from healthy young women show increased proliferation and ER-α expression. At a functional level, estrogen treatment caused EPCs to secrete more growth factors and to integrate into ongoing angiogenesis in a matrigel assay [167]. But estrogens also affect hEPCs reducing senescence through augmentation of telomerase activity [168]. In mice, estrogens have been shown to induce EPC proliferation and migration also through ER and PI3K-dependent pathways [169]. Similar results were seen in spontaneously hypertensive rats where estrogen treatment augmented bone marrow derived EPC differentiation and delayed the onset of senescence [170]. In the same trend, ovariectomized mice showed decreased circulating as well as bone marrow EPC numbers. These effects were completely prevented by estrogen replacement treatment through a decrease in EPC apoptosis. Also, estrogen deficient mice presented increased neointima formation after carotid
artery injury, whereas estrogen treatment accelerated reendothelialization [171].

Other studies have also reported that estradiol increases circulating EPC levels in
mice and improves blood flow recovery while reducing limb necrosis after hind-
limb ischemia [172].

Endothelial progenitor cells are a useful biomarker of cardiovascular disease
[173]. Low circulating EPC numbers and reduced functional capacity have been
associated with increased cardiovascular risk [174]. Reduced EPC migration also
correlates with increased carotid intima to media thickness in humans, an
atherosclerotic indicator [175].

EPCs have been used as a therapeutic tool in over 150 clinical trials [173]. Aoki
et al. found promising results when angioplasty stents were coated with CD-34
antibodies in order to capture circulating EPCs to the damaged area [176].

Several pharmacological approaches that enhance EPC number and function
have been tested. Angiotensin converting enzyme inhibitors and angiotensin
antagonists increase EPC number by reducing oxidative stress and increasing
VEGF release [177, 178]. Statins have been shown to up-regulate NO production through activation of the PI3K/Akt pathway which leads to increased EPC activation in humans [179]. Peroxisome-proliferator-activated receptor gamma agonists reduce EPC senescence, improve adhesion and increase EPC numbers [180]. EPO analogue administration increased EPC circulating numbers by 2.8 time post-acute myocardial infarction [181].

In a rabbit model of atherosclerosis, infusion of EPCs was associated with atherosclerotic plaque regression [182]. In cardiac studies, infusion of EPCs into rats showed poor delivery efficiency: approximately 1% of infused EPCs migrated to the injury site [183]. Directly injecting EPCs in the damaged area may improve clinical outcomes but is unlikely to be possible in all human clinical settings.

Besides nitric oxide, endothelial progenitor cells secrete a vast array of paracrine factors. Rehman et al. showed that EPCs produced vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1), granulocyte colony stimulating factor (G-CSF), and granulocyte/macrophage colony stimulating
factor (GM-CSF) [159]. Other studies showed EPCs secrete factors such as interleukin-8 [184], insulin-like growth factor [185], transforming growth factor β (TGF-beta) [186], platelet derived growth factor (PDGF) [187], anti-angiogenic cytokines such as the tissue inhibitor for the metalloproteases-1 and interferon-inducible protein 10. EPCs are also known to produce neuroregulatory cytokines such as brain-derived neurotrophic factor and leukemia inhibitory factor [188].

**Rationale**

Considering that:

- In-stent balloon angioplasty can lead to restenosis due to the proliferation and migration of vascular smooth muscle cells [153];

- Endothelial cells are known to regulate smooth muscle cell proliferation through the release of nitric oxide [119];

- Estrogens acting through the estrogen receptor (ER)-α on the phosphatidyl-inosito-3-kinase (PI3K)/Akt pathway can enhance nitric oxide production;
• At appropriate levels, nitric oxide has been shown to enhance cell survival [107]:

• Circulating endothelial progenitor cells have the ability to colonize and repair the damaged endothelium;

The goal of the present project is to evaluate the effect of nitric oxide overexpressing early outgrowth EPCs on SMC proliferation and migration. This information will help determine the potential of primed EPCs to prevent restenosis in angioplasty patients.

EPC nitric oxide overexpression will be achieved either by transient transfection of an eNOS plasmid or by estrogen stimulation.
Hypothesis

Endothelial progenitor cell nitric oxide overexpression by eNOS transient plasmid transfection or $E_2$ stimulation will decrease co-cultured vascular smooth muscle cell proliferation and migration through paracrine mediated effects.
Methods

Mononuclear cell isolation

In order to isolate early outgrowth human hEPCs, peripheral blood was drawn from volunteers and 20ml of citrate-buffered blood were mixed with 15ml of phosphate buffered saline (PBS, pH 7.2). Then, 15ml of ficoll (Sigma-Aldrich Histopaque-1083) were carefully overlaid with the diluted blood in a 50ml conical tube and centrifuged at 1160 RCF for 20 minutes at room temperature on a swinging bucket rotor with acceleration and brake set to zero. The mononuclear cells (MNC) found in the interphase were carefully collected with a 10ml pipette and transferred to a new 50ml tube. Cells were washed adding PBS to a total volume of 50ml and rotated at 1160 RCF for 10 minutes with brake. The supernatant was removed and the cell pellet was carefully resuspended in 5ml of PBS. 45ml of PBS were added and centrifuged at 1160 RCF for 10 minutes with brake. The supernatant was discarded and the cell pellet was resuspended in 5ml of EGM-2 endothelial cell medium (Lonza CC-4176). Cell culture plates were
coated with fibronectin (Sigma-Aldrich F0895) and incubated for 30 minutes. The excess fibronectin was removed and the plates were allowed to air dry.

Resuspended MNC were quantified using an improved Hausser-Neubauer counting chamber (Levy Ultra Plane) and seeded on the fibronectin coated dishes. 10^7 cells/60mm dish were placed in an incubator at 37°C 5% CO₂ pressure and the culture medium was refreshed after 4 days.

**EPC transfection**

After the culture medium change of the isolated MNC on day 4, cells were transfected according to the following protocol: 3μl of X-tremeGENE 9 transfection reagent (Roche) were mixed for every 100μl of EBM-2 basal medium (Lonza CC-3156). Plasmid DNA was then added in a 3:1 ratio (μl of transfection reagent:μg of plasmid DNA) and incubated for 15 min at room temperature. The transfection complex was added in a dropwise manner on the cell culture in a 1:10 volumetric ratio (transfection complex:cell medium). Cells were transfected with either dialyzed pVAX1-heNOS plasmid DNA (courtesy of Dr. Nicoletta
Eliopoulos) or pmaxFP-Green-C (Lonza) plasmid as a transfection control. Cells were cultured for 48h after transfection.

17 β-estradiol stimulation

5 days after MNC isolation, cells were treated with 1-100nM E2 (Sigma-Aldrich E8875) overnight. To prevent hCASMC E2-derived stimulation on the co-culture settings, day 6 E2 treated EPCs were washed with PBS, placed in an incubator for 30 min with 10nM of the estrogen receptor inhibitor fulvestrant (Sigma-Aldrich I4409) followed by another PBS rinse and fresh medium change.

Western blotting

10 million day 6 early outgrowth hEPCs cultured in 60mm dishes were harvested with 50μl of cell lysis buffer (HEPES 10nM ph 7.4, Na pyrophosphate 50nM, NaF 50nM, Na₂EDTA 5nM, EGTA 5nM, Triton X-100 0.5%, Na₃VO₄ 2nM, PMSF 1nM, leupeptin 1μg/ml, aprotinin 1μg/ml) per dish, stored in ice and centrifuged at 18000 RCF for 15 min at 4°C. After recovering the supernatant, proteins levels were quantified by Bradford assay (Bio-Rad 500-0006). The appropriate volume
of 6X SDS sample buffer (375mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, 9% 2-
mercaptoethanol, 0.03% bromophenol blue) was added to the maximum amount
of normalized protein sample allowed for a 40μl volume and heated for 5 min at
100°C and then loaded into an 8% polyacrylamide gel. Proteins were transferred
at 35V overnight in a cold room to methanol activated PVDF membrane (Bio-Rad
162-0177). The membrane was blocked for 1 hr at room temperature with
blocking buffer (5% non-fat dry milk in TBS-T) and then incubated overnight with
the appropriate primary antibodies in blocking buffer in a cold room. The
membrane was washed 3 times with TBS-T (50mM Tris pH 7.6, 150mM NaCl,
Tween-20 0.05%) for 5 min at room temperature and then incubated in TBS
(50mM Tris pH 7.6, 150mM NaCl) with the appropriate HRP secondary
antibodies (Santa Cruz goat anti-rabbit sc-2004 or goat anti-mouse sc-2005).
After another 3 TBS-T washes, the membrane was incubated for 1 min with a
western lightning plus-ECL solution (Perkin Elmer NEL105001EA). The
chemiluminescence was measured using Chemidoc XRS+ system (Bio-Rad) and quantified by densitometry using Quantity One software (Bio-Rad).

**BrdU assay**

$10^5$ primary human coronary artery smooth muscle cells (hCASMC) (PromoCell C-12511) per well were seeded in 24 well plates (Corning 3524) in EGM-2 medium. The next day, cells were starved in EBM-2 and 24h later the medium was changed back to EGM-2. Cell culture inserts (BD Biosciences 353095) containing 1M day 6 early outgrowth hEPCs were placed over the hCASMC immediately after BrdU reagent (EMD Millipore 2750 kit) addition at 1:3000 final concentration. After 24hs of co-culture, hEPCs were discarded; hCASMC media was aspirated and cells incubated with 400μl/well of fixing solution for 30 min at room temperature. Following that, cells were washed 3 times with wash buffer for 5 min at room temperature and incubated with 1:200 diluted BrdU detector antibody for 1 hr at room temperature. Cells were washed 3 times more as previously described and then incubated with 1:2000 diluted goat anti-mouse IgG
peroxidase conjugate for 30 min at room temperature. Cells were then washed as described and incubated with 200μl per well of TMB peroxidase substrate for 30 min at room temperature in the dark. Finally, 200μl per well of stop solution were added and the luminescence was measured at 450nm with a fluorescence plate reader (BMG Labtech FLUOstar Optima).

**Wound scratch assay**

hCASMC were divided and 10^5 cells per well were seeded in a 24 well plate in EGM-2 medium. The next day, cells were starved in EBM-2 and 24hs later the medium was changed back to EGM-2. After drawing 2 perpendicular lines at the base of the culture plate under each well, an artificial gap (“scratch”) was produced using a p10 pipette tip parallel to the vertical reference line. Scratched area images were taken above and beneath the horizontal reference line immediately after the scratch (time 0 hr) and at different time points for each condition. Cell culture inserts containing 106 day 6 early outgrowth hEPCs were placed over the hCASMC immediately after images (time 0 hr) were taken with a
Leica DMIL inverted microscope (Leica Microsystems) and a Hitachi HV-F22
(Hitachi Kokusai Electric) progressive scan 3-CCD camera. 30 min later, 0.1μM
PDGF (R&D Systems 120-HD) or the nitric oxide donor S-nitroso-N-
acetylpenicillamine (SNAP, Sigma Aldrich N3398) was added as needed.
hCASMC images were also taken at after 8 and 12 hours of hEPC co-culture.
Images were processed with ImageJ software (NIH) quantifying the cell free area.

**Ki-67 staining**

hCASMC were seeded at an appropriate density on 12mm microscope cover
glass (Fisher Scientific 12-545-82). On the harvest day, cells were rinsed once
with PBS and fixed with 2% paraformaldehyde (PFA) for 15 min at 37°C, and
then washed 3 times with PBS for 5 min at room temperature. Cells were further
permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature and
another 3 PBS washes were performed. Cells were blocked with 3% BSA in PBS
overnight at 4°C, then rinsed with PBS and incubated with 1/50 diluted rabbit anti-
Ki-67 antibody (Abcam ab16667) in PBS for 90 min at room temperature. Cells
were washed 3 times with PBS for 5 min at room temperature and then incubated in the dark for 1 hr with a 1/500 Alexa Fluor 555 goat anti-rabbit fluorescent secondary antibody (Invitrogen A-21428) in PBS. Afterwards, cells were washed 3 times with PBS for 5 min at room temperature in the dark and microscope cover glasses were mounted on slides (VWR 48311-703) containing 10μl of mounting medium with DAPI (Vector Laboratories H-1500). Images were taken using a Leica DM2000 fluorescent microscope (Leica Microsystems) and an Infinity3-1UC color camera (Leica Microsystems). Ki-67 positive cells were quantified as a percentage of total DAPI stained nuclei and normalized against either untreated hEPCs or transfection control hEPCs (basal conditions).

**Flow cytometry**

4 million monocytes per well (isolated as previously described) were seeded in 6 well plates (Corning 3516). On day 6, cells were washed with PBS and detached using 500μl of TrypLE (Invitrogen 12605-010) for 5 min at 37°C. Then, cells were suspended in 1ml of PBS 3% FBS and centrifuged at 1160 RFC for 10 min. For
the Annexin V (AV; BD Biosciences 560931) and propidium iodide (PI; BD Biosciences 51-66211E) staining, the supernatant was aspirated and cells were resuspended in 300μl of 1X Annexin V binding buffer (BD Biosciences 51-66121E) and transferred to flow cytometry test tubes (Falcon 2058). Cells were incubated in the dark for 15 min with 1μl of FITC Annexin V and 2μl of propidium iodide staining solution per sample tube. For compensation purposes, a PI only, an AV only and an unstained cell tube were also prepared. Apoptosis was assessed using a FACSCalibur flow cytometer (BD Biosciences) considering all AV and PI double positives as apoptotic cells.

For the cleaved caspase-3 staining cells were resuspended in 100μl of PBS 2% FBS after the first centrifugation. In order to increase labelling specificity, cells were incubated with 10μl per tube of human FcR blocking reagent (Miltenyi Biotec 120-000-442) for 30 min at room temperature. Then, cells were centrifuged as previously described and resuspended in 250μl of a fixation/permeabilization solution (BD Cytofix/Cytoperm Fixation/Permeabilization
Kit 555714). After 20 min incubation at 4°C, cells were washed by centrifugation with 1ml of BD Perm/Wash buffer and resuspended in 100μl of the same buffer in order to keep them permeabilized. Cleaved caspase-3 antibody (Cell Signaling 9669S; rabbit) was added to each condition at a concentration of 1/50 and incubated in the dark for 30 min at 4°C. To estimate the nonspecific binding of target primary antibody due to Fc receptor binding or other protein interaction, a separate condition was incubated with 1/50 of rabbit IgG isotype control (Cell Signaling 4340S). Finally, cells were centrifuged and the resulting pellet was resuspended in 300μl of PBS. Cleaved caspase-3 positive cells were considered as undergoing apoptosis and were quantified using a FACSCalibur flow cytometer.

Results were analyzed using CellQuest Pro software (BD Biosciences).

**NO probe**

1 million monocytes (isolated as previously described) were seeded on 12mm fibronectin coated microscope cover glasses. On day 6, DAF-FM diacetate
(Molecular Probes D-23842) was added to a final concentration of 1μl for 1 hour at 37°C. Then, the medium was removed and a 10 min PBS wash was performed at 37°C. Cells were fixed with 2% PFA for 15 min at 37°C, and then washed 3 times with PBS for 5 min at room temperature in the dark. Microscope cover glasses were mounted on slides containing 10μl of mounting medium with DAPI. Images were taken using an Infinity 3 Leica fluorescent microscope. NO positive cells were quantified as a percentage of total DAPI stained nuclei and normalized against untreated hEPCs (basal condition).
Results

Effect of an NO donor on SMC proliferation and migration

Nitric oxide has been shown to decrease proliferation in cultured rat smooth

muscle cells [119]. To determine if EPCs have a similar effect, hCASMC were

serum starved for 24h. The medium was changed to EGM-2 and BrdU reagent

was added. Following this, the cells were treated with the NO donor SNAP and/or

24-well inserts were seeded with $10^6$ hEPCs placed on the hCASMC wells. After

24hs of incubation, hEPCs were discarded, hCASMC were rinsed and the

amount of BrdU incorporated was measured by fluorescence.

Results show that hCASMC proliferation decreased in a dose dependent manner

when increasing quantities of the SNAP were used (Figure 1) compared to the

untreated control. Co-culture of hEPC with the SNAP treated hCASMC didn’t

have a supplemental effect whereas the co-culture with hEPCs alone didn’t show

a significant effect on hCASMC proliferation.
To examine the effect of the NO donor on hCASMC migration, a wound scratch assay was performed on the hCASMC. Brightfield pictures of the scratch area were taken 8hs later using an inverted microscope and the migration area was quantified using ImageJ software.

As can be seen on Figure 1, HCASM migration was decreased by SNAP treatment, and no supplemental effect could be seen when co-cultured with native hEPC.

These results indicate that NO decreases both hCASMC proliferation and migration but the addition of native hEPC fails to have an additive effect. However, hEPC co-culture without SNAP has no visible consequences on hCASMC under the current experimental setting.

**eNOS overexpression is associated with increased NO production**

In order to achieve NO overexpression, hEPC were transfected with either human eNOS plasmid (EPC eNOS) or a transfection control plasmid (EPC TC). Cells were lysed 48hs later.
As seen in Figure 2A, there was a three-fold increase in both the total and the activated (phospho S1177) eNOS forms in the eNOS transfected hEPCs compared to the control plasmid transfected cells.

To assess nitric oxide production, hEPCs were incubated during 1 hour with DAF-FM diacetate 48h after plasmid transfection. The proportion of NO positive cells was calculated for each field. Figure 2B shows an increase in NO production similar to the one observed for the eNOS protein isoforms observed by western blot.

These results would indicate that eNOS plasmid transfection results in increased total eNOS as well in the activated eNOS isoform protein levels. Following eNOS transfection, a rise in NO production was observed suggesting a direct correlation between eNOS transfection, eNOS overexpression and nitric oxide production.

**eNOS overexpression increases EPC survival**

While increased levels of NO have been associated with positive physiological changes in several biological models [27, 31, 93], excessive levels have been
connected with increased apoptosis [29, 103, 106]. To determine whether the NO overexpression achieved in the present project had deleterious effects on the survival of the hEPCs, apoptotic levels were evaluated by flow cytometry and western blotting.

To determine apoptosis levels by flow cytometry, hEPCs transfected with either an eNOS or control plasmid were stained with either cleaved caspase-3 antibody or with Annexin V (a cell surface protein found in apoptotic cells) and propidium iodide (PI; an intercalating agent that binds to nucleic acids, used to asses cell integrity). Double positive cells were quantified using a FACSCalibur flow cytometer.

The results show a significant decrease in cleaved caspase 3 staining for eNOS transfected hEPCs compared to the transfection control (Figure 3A) and a trend towards less Annexin V/PI staining for eNOS transfected hEPCs (Figure 3B).

EPC survival was also evaluated measuring B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) levels by western blotting. Bcl-2 has been shown to
have antiapoptotic activity whereas Bax promotes apoptosis competing with Bcl-2 [189]. Figure 3C shows a significant increase in the Bcl-2/Bax ratio for the eNOS transfected EPCs suggesting an increased protection against apoptosis compared to the control condition.

Summarizing, both Annexin V/PI staining and Bcl-2/Bax western blotting indicate that eNOS transfected hEPCs have enhanced protection against apoptosis.

**eNOS overexpressing hEPCs decrease hCASMC migration but not proliferation**

Even though published results show that nitric oxide decreases smooth muscle cell migration [121], native hEPCs didn’t seem to have an effect on hCASMC under our experimental settings (Figure 1). In order to test whether eNOS overexpressing hEPCs could modulate hCASMC migration, hCASMC were co-cultured with transfected hEPCs for 12hs.

As seen in Figure 4A, no significant difference could be seen between the migrated area of hCASMC alone and in the presence of native hEPCs. On the other hand, hCASMC co-cultured with eNOS overexpressing hEPCs migrated
less than hCASMC co-cultured with control transfected hEPCs. These results would indicate that nitric oxide released by EPCeNOS effectively reduces hCASMC migration through paracrine effects.

Besides migration, nitric oxide has been reported to modulate smooth muscle cell proliferation [117]. To evaluate proliferation, Ki-67 staining was performed on hCASMC. Ki-67 is a nuclear protein present during all active phases of the cell cycle but absent from resting cells, and therefore used as a cellular marker of proliferation.

hCASMC plated on cover slips were co-cultured with eNOS transfected, control transfected or native hEPCs during 24hs, and the percentage of Ki-67 positive cells was quantified.

As seen in Figure 4B, the amount of Ki-67 positive hCASMC didn’t significantly decrease when in the presence of hEPCs compared to hCASMC alone. This would suggest that hEPCs are not able to diminish hCASMC proliferation through paracrine effects.
In the same trend, when hCASMC were cultured in the presence of eNOS overexpressing hEPCs, no significant proliferation difference could be seen on the hCASMC compared to when co-cultured with control transfected hEPCs (Figure 4B).

These results suggest that neither native hEPCs nor eNOS transfected hEPCs have the ability to downregulate hCASMC proliferation in vitro under the current conditions.

**Estrogen induces NO production in hEPCs**

It has been reported that nitric oxide production can be modulated by estrogen stimulation. Estrogens act through the estrogen receptor (ER)-α to activate the c-SCR/PI3K/Akt pathway [74] that ultimately leads to the activation of eNOS by phosphorylation of serines 615 and 1177.

In order to confirm in the present model that the nitric oxide modulation was due to E₂ stimulation and was not vehicle-induced, a concentration curve was performed. It was determined that an optimal 17β-estradiol (E₂) dose of 10⁻⁹M
was appropriate to maximize NO production while preventing vehicle cross-stimulation (Figure 5A).

To determine whether estrogen stimulated hEPC could be used to regulate hCASMC proliferation and migration, total and phosphorylated eNOS as well as nitric oxide production was measured after E2 stimulation.

As seen in Figure 5B phospho eNOS was increased in the $10^{-9}\text{M}$ E2 stimulated hEPCs compared to the vehicle induced cells. This result correlates with the increase in nitric oxide production observed after DAF-FM staining in the E2 stimulated hEPCs (Figure 5C). Summarizing, E2 stimulated hEPCs have increased phospho and total eNOS levels and enhanced nitric oxide production.

**Estrogen increases hEPC survival**

As previously mentioned for the eNOS overexpressing hEPCs, it was important to determine if the NO levels of the E2 stimulated cells influenced their survival.

Figure 6 shows that E2 stimulated hEPCs have higher Bcl-2 to Bax ratio than
vehicle stimulated or native cells. This would indicate that E2 confers a protective
effect on hEPCs possibly through the upregulation of nitric oxide production.

**E2 stimulated hEPCs decrease HCASM migration but not proliferation**

We showed that eNOS overexpressing hEPCs can modulate hCASMC migration.

To determine whether E2 stimulated hEPCs could have a similar effect, overnight
E2 stimulation was performed on hEPCs. To prevent E2 derived cross-stimulation,
hCASMC were incubated for 30 min with 10nM of the estrogen receptor inhibitor
fulvestrant (Sigma-Aldrich I4409) prior to co-culture. After performing the scratch
assay, hCASMC were incubated in the presence of E2 stimulated hEPC and
photos were taken at 0, 8 and 12h. The cell-free area was quantified using
ImageJ software. Figure 7A shows that, unlike their native counterparts, E2
stimulated hEPCs are able to decrease hCASMC migration, but have no effect on
hCASMC proliferation, as seen by Ki-67 staining (Figure 7B). This result
suggests that hCASMC migration but not proliferation can be modulated by E2
stimulated hEPCs, presumably through NO release.
eNOS and NO production in E2-stimulated eNOS overexpressing hEPCs

The previous results show that hEPC eNOS and NO levels were upregulated after eNOS transfection or E2 stimulation. To determine whether a synergistic effect could be observed, hEPCs were both transfected with an eNOS plasmid and stimulated with $10^{-9}$ E2.

As seen in Figure 8A, an increase in phospho-eNOS was observed in control transfected hEPCs stimulated with E2 and in eNOS-overexpressing hEPCs, but no further effect of E2 was observed in eNOS-overexpressing hEPCs. These effects were verified in cells incubated with the NO indicator DAF-FM acetate (Figure 8B). Using this assay, an increase in NO levels could be observed on eNOS transfected E2 stimulated hEPCs (EPCeNOS+E2) compared to control transfected E2 stimulated hEPCs (EPCTC+E2), suggesting a potential synergistic effect of eNOS transfection+E2 stimulation on NO production.
**Apoptosis levels on E₂ stimulated eNOS transfected hEPCs**

We showed an increase in survival in eNOS transfected or E₂ stimulated hEPC (Figures 3 and 6). Figure 9A confirms a significant decrease in cleaved caspase-3 in both control transfected E₂ stimulated hEPCs (EPCTC+E₂) and eNOS transfected DMSO stimulated hEPCs (EPCeNOS+DMSO) compared to the control transfected DMSO stimulated hEPCs (EPCTC+DMSO). Moreover, a mild additive effect could be seen on eNOS transfected E₂ stimultated hEPCs.

We found that Bcl-2/Bax results mimic our cleaved caspase-3 findings, where EPCTC+E₂ and EPCeNOS+DMSO survival is increased compared to our control, but no additive effect could be seen when combining eNOS transfection and E₂ stimulation (Figure 9).

These results support our previous findings showing a decrease in apoptosis in E₂ or eNOS transfected hEPCs but show little or no synergistic effect when cells were subject to both treatments.
Effect of eNOS transfected E2 stimulated hEPCs on hCASMC migration

To determine if eNOS transfection and E2 stimulation of hEPCs acts on hCASMC migration, a wound scratch assay was performed.

Figure 10A shows that after 8 hours of co-culture, a significant decrease in hCASMC migration could only be observed when in the presence of control transfected E2 stimulated hEPCs (EPCTC+E2). After 12 hours of co-culture, eNOS transfected DMSO stimulated hEPCs (EPCeNOS+DMSO) significantly reduced hCASMC migration as well. No further effect could be seen when hEPCs were transfected with eNOS and stimulated with E2. Similarly, no significant difference in proliferation rates could be appreciated among the different conditions using Ki-67 staining. These results confirm that E2 or eNOS transfection inhibit hCASMC migration but no synergistic effect could be observed.
Effect of eNOS transfected E₂ stimulated hEPC on PDGF stimulated hCASMC protein kinase pathways

The platelet-derived growth factor (PDGF) is a potent mitogen for smooth muscle cells. PDGF acts through the PDGF receptor (PDGFR) and upon binding, PDGFR dimerizes and initiates a cascade of signals that lead to the activation of the MAP-kinase ERK pathway [190]. PDGF can also stimulate the focal adhesion kinase (FAK). FAK regulates cell growth and survival through activation of the ERK pathway, among others [191].

To determine whether hCASMC MAP-kinase activation could be modulated by eNOS transfected or E₂ stimulated hEPCs, hCASMC were co-cultured with hEPCs for 1 hour after which the hCASMC were lysed.

We saw that PDGF increased ERK 1/2 activity in hCASMC but had no effect on FAK activity. Also, co-culture of hCASMC with untreated hEPCs, or hEPCs either transfected with eNOS, stimulated with E₂, or both, did not diminish ERK 1/2 activation by PDGF (Figures 11-13). These results suggest that hCASMC PDGF
stimulation is able to regulate ERK 1/2 activation but not FAK. Finally, co-culture
with native, eNOS transfected, E₂ stimulated, or both, hEPCs was not able to
modify native or PDGF induced hCASMC ERK 1/2 or FAK levels. This would
indicate that hEPC NO production is not able to modulate the aforementioned
proteins in hCASMC under the current conditions.

**Statistical Analysis**

All data presented as mean ± standard error of the mean. Data were analyzed by
ANOVA, and intergroup comparisons performed by Student’s t-test. Probability
values of \( p \leq 0.05 \) were considered to be statistically significant.
Conclusion and summary

In the present work, we showed that hCASMC proliferation and migration are decreased in the presence of the NO donor SNAP in a dose dependent manner. Co-culture with native hEPCs didn’t show a supplemental effect on SNAP-stimulated hCASMC and no effect was observed when hEPCs were co-cultured with untreated hCASMC. In the same line as previous studies [119, 121], our results indicate that smooth muscle cell proliferation and migration are sensitive to nitric oxide. On the other hand, hEPC co-culture didn’t have an effect on hCASMC proliferation suggesting that, under the current experimental settings, native hEPC NO production is not sufficient to affect the neighbouring cells.

Following hEPC eNOS transfection, an increase in both total and phosphorylated eNOS levels as well as a higher NO release could be observed. At the same time, hEPC apoptotic levels decreased, as seen by a decrease in cleaved caspase-3 levels and a rise in Bcl-2/Bax. These results show that the rise of eNOS and phospho eNOS protein levels correlate with the increased levels of
nitric oxide observed, and that these levels were high enough to improve cell survival while avoiding the rise in apoptosis sometimes associated with excessive NO production [101].

Moreover, we showed, hCASMC proliferation was reduced using the NO donor SNAP, but neither native nor eNOS transfected hEPCs were able to mimic this effect. This suggests that higher NO levels are required to modulate hCASMC proliferation in vitro.

eNOS can be activated by phosphorylation of serine 1177 by an estrogen mediated pathway. Thus, unsurprisingly, we observed a similar set of results when we stimulated hEPCs with E2. Both the phosphorylated eNOS isoform and total NO levels were upregulated in E2 stimulated hEPCs while, at the same time, apoptosis was decreased. Much like their eNOS transfected counterparts, E2 stimulated hEPCs reduced hCASMC migration but not proliferation.
When hEPCs were both eNOS transfected and E₂ stimulated, a synergistic effect could be seen on NO production but not in apoptosis levels, nor in eNOS or phospho-eNOS protein quantities.

hCASMC migration was not further decreased when co-cultured with eNOS transfected E₂ stimulated hEPCs and hCASMC proliferation remained unchanged among all experimental conditions.

PDGF stimulation of hCASMC co-cultured with native, eNOS transfected or E₂ stimulated hEPCs showed either a trend or an increase in Erk 1/2 activation but no effect on FAK. The different hEPC conditions didn’t produce a significant difference on either MAP-kinase phosphorylation level.

These results suggest that PDGF activated pathways are not directly affected by NO emanating from hEPCs.

In order to prevent restenosis after angioplasty, a first generation of drug eluting stents (DES) were designed. Stents were covered with immunosuppressive agents such as rapamycin in order to limit SMC proliferation and migration.
Unfortunately, these drugs not only prevented SMC proliferation but also slowed vascular re-endothelialization by promoting EPC apoptosis [192]. The possibility of coating DES with SMC proliferation inhibiting agents that at the same time accelerate vascular repair has been proposed [192]. This approach has been used successfully in animal models using VEGF-2 growth factor gene-eluting stents [193]. Another interesting strategy involves the capture of circulating EPCs using stents coated with antibodies or peptides that bind to their membrane receptors [194, 195]. Since nitric oxide can’t be applied directly over the stent due to its physicochemical properties, EPC mediated NO delivery could be a “natural” approach to tackle SMC proliferation while enhancing re-endothelialization. Stent-delivered hEPCs could have the potential not only to release NO and control SMC, as shown in our study, growth but also to express molecules or secrete factors that could recruit circulating EPCs to the site of injury further increasing endothelial recovery.
Besides DES, systemic medication administration could also influence the balance between re-endothelialization and neointimal proliferation. Some publications suggest that statins promote the number of circulating EPCs [196] while others find no significant change [161]. Thiazolidinediones have also been reported to increase EPC numbers while reducing EPC apoptosis in a PI3K dependent manner [197]. These drugs have the potential to reduce restenosis.

Many factors may have influenced the variability observed in some of the results in this project. Among them, we have to consider cell population composition. EPCs are derived from pluripotent stem cells that are able to differentiate into several lineages including macrophages and smooth muscle cells [185]. For example, cultured endothelial colony-forming cells implanted *in vivo* have been shown to form *de novo* blood vessels in a mouse model, an unwanted feature in malignant diseases but an attractive feature in the context of myocardial ischemia [198]. EPCs are believed to derive mainly from the bone marrow but they have also been seen to originate from the spleen, adipose tissue and vessel wall [199-
Due to the fact that many of the markers used to identify EPCs are not specific to this cell type, there is not a clear consensus on EPC characterization [203]. Even though the mononuclear cell isolation protocol employed herein is designed to select only hEPCs, its efficiency most likely is not 100%. Previous characterization done in our laboratory by Talin Ebrahimian quantifying the uptake of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine acetylated low-density lipoprotein (LDL) and binding of endothelial-specific lectin showed that more than 80% of the cells had the EPC phenotype. This leaves a significant proportion of the isolated cell population uncharacterized which could represent an important source of variation.

Another factor that could have contributed to result fluctuation was blood donor source. Even though all donors were young and had no major health problems, it is known that EPC function and numbers depends on many factors such as age, gender, life style (physical activity levels, smoking habits, diet) among others.
VEGF, MMP-9, soluble kit-ligand, stromal cell derived factor 1, G-CSF and shear stress are factors known to favour EPC differentiation [204]. Post-angioplasty, EPC differentiation might also depend on the lesion size. A larger denuded area could mean a lower endothelial cell to SMC ratio which could hinder re-endothelialisation whereas smaller lesions would have a better micro-environment that could allow for a faster recovery.

Among the limitations of the current model, Ignarro et al. have suggested the possibility that NO itself is not the diffusing agent, but an intermediate compound formed when NO interacts with sulfhydryl groups in the extracellular domain [205]. It would be this longer lasting intermediate compound the responsible for the observed physiological effects [206]. For example, it has been reported that SMC and platelets respond to the NO derivates S-nitrosylthiols and to NO itself similarly. It has this been proposed that in the blood vessels of the central nervous system the effector molecule is not NO but the S-nitrosylthiols [207]. Nevertheless, the most accepted opinion is that NO actually causes SMC
relaxation and S-nitrosothiols are only intermediate compounds or reaction
products [208]. Understandably, our simplified cell model is unable to reproduce
the numerous circulating factors and interactions present in the vasculature.

Therefore it would be of interest to see whether these results could be
reproduced or even enhanced in vivo.

It should also be pointed out that regenerated endothelial cells don’t have the
same characteristics as the native endothelium. Regenerated cells appear to be
dysfunctional which favours the occurrence of atherosclerosis [209]. Several
phenotypic changes occur on regenerated endothelial cells including the
presence of enlarged cells, multinucleated cells, appearance of early
senescence, reduced eNOS activity, increased ROS production, greater modified
LDL cholesterol uptake, increased oxidized LDL production, and accelerated
apoptosis [210].

Even though most studies associate higher circulating EPC numbers with better
cardiovascular health [166], Pasceri et al. have found that patients with higher
endothelial cell counts had a higher in-stent restenosis compared to lower cell counts [211]. This may be partly due to the possibility that bone marrow progenitors can also differentiate into smooth muscle cells thus aggravating the restenosis [212].

Summarizing, we found that native early outgrowth hEPCs have no effect on hCASMC migration or hCASMC proliferation. However, NO overproducing hEPCs (either eNOS transfected or E_2 stimulated) have increased survival and decrease hCASMC migration but have no further effect on hCASMC proliferation. Our results suggest that eNOS overexpression and or E_2 stimulation in early outgrowth hEPCs increases their survival and enhances their capacity to modulate human coronary artery SMC migration through paracrine effects.
Fig. 1. Effect of an NO donor on SMC proliferation and migration
SMCs were stimulated with increasing concentrations of the NO donor SNAP (s-nitroso-N-acetylpenicillamine) and/or co-cultured with hEPCs.
(A) Proliferation was assessed by BrdU incorporation and reported as the percentage of SMC BrdU positive cells. (B) SMC migration was determined by wound scratch as the percentage of migrated area. (C) Representative images of the SMC migration were included.
Fig. 2. eNOS overexpression by plasmid transfection

(A) Total and phosphorylated eNOS expression of control transfected (EPCTC) or eNOS transfected (EPCeNOS) hEPCs was assessed by western blotting. (B) Nitric oxide production was measured by fluorescent microscopy as the percentage of DAF-FM positive (green)/DAPI stained cells.
Fig. 3. Effect of eNOS overexpression by plasmid transfection on EPC survival
hEPC apoptosis levels were measured by flow cytometry as the percentage of either active caspase III (A) or annexin V/PI (B) stained cells, or by western blotting as the ratio between the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins (C).
Fig. 4. Effect of eNOS overexpression by plasmid transfection on EPC migration and proliferation

(A) SMC migration was assessed by wound scratch assay in the presence or absence of hEPC; the result was as expressed as the percentage of the migrated area. (B) Proliferation of both native and hEPC co-cultured SMC was evaluated by Ki67 immunofluorescent microscopy and expressed as the percentage of positively stained cells.
Fig. 5. E2-stimulated intact EPC
A dose-response curve was performed to determine the optimal E₂ concentration ($10^{-9}$) at which the NO production was the highest (A). Levels of the active (phosphorylated) eNOS isoform were assessed by western blotting in E₂ or vehicle (DMSO) stimulated hEPCs using the reference concentration value (B). NO production was expressed as the ratio of DAF-FM (NO probe) positive cells to DAPI stained nuclei (C).
Fig. 6. E2-stimulated intact EPC survival

eNOS transfected hEPC survival was evaluated by flow cytometry quantification of active caspase III positive cells (A) or Annexin V/PI staining (B) and also by western blotting expressed as the ratio between the levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax (C).
Fig. 7. Effect of E₂-stimulated intact EPC on SMC migration- proliferation
In the presence of E₂ stimulated hEPCs, SMC migration was evaluated by wound
scratch assay (A) while SMC proliferation was assessed as the ratio of Ki-67 positive
to DAPI stained cells (B).
Fig. 8. E₂-stimulated eNOS transfected hEPCs.
eNOS and phospho-eNOS levels were quantified by western blotting in E₂ stimulated 
eNOS transfected hEPCs (A) while NO production was measured as the ratio of 
DAF-FM positive to DAPI stained cells (B).
Fig. 9. E2-stimulated eNOS-overexpressing EPC survival
eNOS overexpressing and/or E_2_ stimulated hEPC survival was evaluated by flow
cytometry quantification of active caspase III positive cells (A) as well as by western
blotting expressed as the ratio between the levels of the anti-apototic protein Bcl-2
and the pro-apoptotic protein Bax (B).
Fig. 10. Effect of E2-stimulated eNOS overexpressed EPC on SMC migration and proliferation

(A) SMC migration was assessed by wound scratch assay in the presence E2 stimulated and/or eNOS transfected of hEPC; the result was as expressed as the percentage of the migrated area.

(B) Proliferation of hEPC co-cultured SMC was evaluated by Ki67 immunofluorescent microscopy and expressed as the percentage of positively stained cells.
Fig. 11. Effect of eNOS overexpression by plasmid transfection in EPCs on ERK1/2 and FAK Activation in SMC

SMC P-ERK-1/2 (A) or P-FAK (B) expression was evaluated by western blotting (B) when co-cultured with transfected hEPCs in the presence or absence of PDGF. All conditions were normalized against the control group (SMC-VEH).
Fig. 12. Effect of E2-stimulated intact EPCs on ERK1/2 and FAK activation in SMC

SMC P-ERK-1/2 (A) or P-FAK (B) expression was evaluated by western blotting (B) when co-cultured with E2-stimulated hEPCs in the presence or absence of PDGF. All conditions were normalized against the control group (SMC-VEH).
Fig. 13. Effect of E2-stimulated eNOS-overexpressed EPCs on ERK1/2 and FAK activation in SMC

SMC P-ERK-1/2 (A) or P-FAK (B) expression was evaluated by western blotting (B) when co-cultured with E2 stimulated and/or eNOS transfected hEPCs in the presence or absence of PDGF. All conditions were normalized against the control group (SMC-VEH).
References

5. Dronsfield, A. *Joseph Priestley and Nitrous Oxide*.
10. Team, C. *nitric oxide*. 25 August 2011; Available from: [https://www.ebi.ac.uk/chebi/searchId.do?chebiId=16480](https://www.ebi.ac.uk/chebi/searchId.do?chebiId=16480).
22. *Fourth Assessment Report (AR4) by Working Group 1 (WG1), Chapter 2 "Changes in Atmospheric Constituents and in Radiative Forcing" which contains information on global
warming potential (GWP) of greenhouse gases. 2007: Intergovernmental Panel on Climate Change.


115


