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Title

Three protective targets in vascular system through the interaction with NO and H_2S: GILZ, DHEA and Opa1 protein

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LIST OF ABBREVIATIONS

Abbreviations	Definition
ACh	Acetylcholine
AR	Androgen receptor
Arg-1 and Arg-2	Arginase 1 and Arginase 2
ATP	Adenosine-5'triphosphate
BH_4	(6R-) 5,6,7,8-tetrahydro-L-biopterin
BH_2	7,8-dihydrobiopterin
BAEC	Bovine aorta endothelial cells
Ca^{2+}	Calcium
CAT	Cysteine aminotransferase
Cav-1	Caveolin-1
CBS	Cystathionine-beta-synthase
CCRC	Cumulative concentration-response curve
cGMP	3',5'-cyclic guanosine monophosphate
COX-1 and COX-2	Cyclooxygenase
CSE	Cystathionine-gamma-synthase
CTR	Control
CVD	Cardiovascular diseases
DEX	Dexamethasone
DG	Diacylglycerol
DHEA	Dehydroepiandrosterone
Drp1	Dynamin-related protein 1
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
ECL	Enhanced chemiluminescence
eNOS	Endothelial nitric oxide enzyme
E_2	Estrogen
ECT	Electron transfer chain
ERα	Estrogen receptor
ERK1/2	Extracellular signal-regulated kinases
FAD	Flavin adenine dinucleotide
Fis1	Fission 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GILZ	Glucocorticoid Induced Leucine Zipper
GC	Glucocorticoid
Gly	Glybenclamide

GR	Glucocorticoid cytoplasmatic receptor
GPCR	Receptor coupled to G protein
GTP	Guanosine triphospohate
5-HT	Hydroxytryptamine
HOCl	Hydrochloride acid
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HUVEC	Human umbilical vein EC
ICAM-1	Intercellular adhesion molecule 1
iNOS	Inducible nitric oxide synthase enzyme
INDO	Indometacin
IP_3.	Inositol triphosphate
K_{ATP}	ATP-dependent potassium
KCl	Potassium chloride
KO	Knockout
L-Arg	L-arginine
L-Cys	L-cysteine
L-NAME	L-NG-nitro-arginine methyl ester
L-NIO	N^5-(1-Iminoethyl)-L-ornithine dihydrochloride
Mfn1	Mitofusin-1
Mfn2	Mitofusin-2
MLC	Myosin light chain
MLC kinase	Myosin light chain kinase
MLC phosphatase	Myosin light chain phosphatase
3-MST	Third 3-mercaptopyruvate sulfur transferase
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial ROS
NADPH	Nicotinamide-adenine-dinucleotide phosphate
NaHS	Sodium hydrosulfide
$NaNO_2$	Sodium nitrite
Na_2S	Sodium sulfide
NCD	Noncommunicable diseases
Nil	Nilutamide
NO	Nitric oxide
NO_3^-	Nitrate
NO_2^-	Nitrite

NOX_2	NADPH oxidase 2
Nrf1	Nuclear respiratory factor
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Opa1	Optic atrophy type 1
O_2.	Oxygen
$O_2^{-\bullet}$	Superoxide anion
$^{\bullet}OH$	Radical hydroxyl
$ONOO^-$	Peroxynitrite
OXPHOS	Oxidative phosphorylation
PAG	Propargylglycine
PE	Phenylephrine
peNOS	Phosphorylated eNOS
PGC-1α	Peroxisome proliferation-activated receptor gamma co-activator 1α
PGI_2	Prostacyclin
PGE_2	Prostaglandin
PIP_2	Phospholipid phosphatidylinositol biphosphate
PKC	Protein kinase C
PKG	Protein kinase
PSS	Physiological salt solution
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SIRT1	Silent information regulator 2 homolog 1
SMCs	Smooth muscles cells
SOD	Superoxide dismutase
T	Testosterone
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor
TNF-α	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule
WT	Wild type

ABSTRACT

Cardiovascular diseases (CVDs) are the most important non-transmissible disease present worldwide. An endothelial dysfunction is often associated to CVD as a possible cause or concomitant cause. Nitric oxide (NO) and hydrogen sulfide (H_2S) are two gasotransmitters playing an important role in the control of vascular tone. This thesis aimed to focus on potential protective targets for CVD. In particular, the functional and biochemical exchanges in vasculature were studied. The first one target is Glucocorticoid Induced Leucine Zipper (GILZ) peptide, a widely demonstrated anti-inflammatory molecule, to be used as alternative therapeutic approach of glucocorticoid (GC) treatment, avoiding one of well-known associated vascular side effect, the arterial hypertension. In general GCs treatments are increasingly implicated in the pathogenesis of obesity, metabolic syndrome, arterial hypertension. Thus, there is a need for searching both GCs targeted therapies and additional therapeutic agents/strategies for reducing undesired side effects of chronic GCs treatment. We demonstrated by functional and biochemical study that $GILZ^{-/-}$ mice have a reduction of NO and H_2S signaling either in the aorta and plasma. Our evidence suggests that GILZ pathway has also a role in the regulation of vascular tone, supporting the hypothesis that exogenous synthetic GILZ peptide could represent a valid alternative in the replacement of GC. A previous publication demonstrated that the co-administration of dehydroepiandrosterone (DHEA) with dexamethasone resulted in reduction of oxidative stress in kidney-cortex, attenuation of albuminuria and normalization of glutathione redox state, in rabbit. The authors conclude that DHEA might limit several undesirable renal side effects during chronic GC treatment and might be particularly beneficial for the therapy of patients with glucocorticoid induced diabetes. In this regard, in the second part, we focused on the DHEA, a precursor of sex hormone, that decreases with age and seems to have a protective vascular effect. We demonstrate that the relaxant effect of DHEA involves the formation of H_2S and NO. We addressed this issue by using mice vessel and cell culture; functional and biochemical assay were performed using CD1 mice and

bovine aorta endothelial cells (BAEC). In the last part the involvement of the endothelium as the major responsible of CVD has been addressed with particular attention to the role of a specific protein involved in mitochondria functions. In particular, the third target is the optic atrophy type 1 (Opa1) a key protein that allows the mitochondria fusion. We studied knockout mice where Opa1 has been silenced exclusively in the endothelial cells (EC) (EC-Opa1$^{-/-}$). In old EC-Opa1$^{-/-}$ mice kidney we observed an increase of oxidative stress and an increase of protein nitrosylation with an augmentation of caveolin expression suggesting un uncoupling of endothelial nitric oxide synthase enzyme (eNOS), the enzyme of synthesis of NO. We also demonstrated that in EC-Opa1$^{-/-}$ old mice there is a reduction of cystathionine-gamma-synthase (CSE) protein expression, one of the main enzymes involved in the bio-synthesis of H_2S in vasculature. On the other hand, an increase of inflammatory protein in EC-Opa1$^{-/-}$ kidney and mesenteric artery in adult mice was observed. Thus, Opa1 is important in EC homeostasis and might protect the vascular tree in target organs such as the kidney with a role in aging associated vascular diseases.

In conclusion, all the three targets, seems to be associate in a different way to gas transmitters production. In particular we observed a correlative relation with NO and H_2S, confirming the key role in cardiovascular disease, underlying, once again, as important are these gasotransmitters in the control of vasculature function.

CHAPTER I
CARDIOVASCULAR SYSTEM

The cardiovascular system has a vital role in bringing oxygen and nutriment to all organs to support the metabolism of tissue. It is formed by two different parts, systemic circulation and lung circulation. In the first one, the heart pumps the blood in the longest artery which is the aorta, after passing into the artery of less caliber, the resistance artery, to finish in the arterioles and then in the capillary (figure 1A). It is at this level that happens the important exchange between vessel and tissue, from the blood nutriment and oxygen to tissue, and from tissue CO_2 and metabolic waste products to vessel. The blood enters then into the veins which allows its return to the right atrium of the heart and then into the pulmonary artery; thus, the systemic circulation becomes lung circulation, where the blood rich in CO_2 becomes rich in O_2. The arterial tissue is formed by three layers: the tunica adventitia, the media, and the intima (figure 1B). The adventitia contains mostly connective tissues made of collagen fibers, fibroblasts, and progenitor cells. In the tunica adventitia are present sympathetic efferent nerves and, in the largest arteries, the vasa vasorum that feed with oxygen and nutriment the vessels walls. The external elastic lamina separates the adventitia and the media. The tunica media is mainly composed of smooth muscles cells (SMCs) surrounded by elastic and collagen fibers. Large arteries called conductance or elastic arteries such as the aorta, are characterized by a large proportion of elastic fibers that allows the arteries to dilate during the systole and to contract during the diastole. Muscular arteries follow the elastic arteries. They contain more SMCs which allow them to control the blood flow to the tissues. Finally, the smallest arteries, also called resistance arteries (as mesenteric artery), contain a lower number of SMC layers and their external and internal elastic lamina is less pronounced. The tunica intima is composed of a monolayer of endothelial cell (EC) that lines the vascular lumen, and between the media and the intima, there is a limited elastic intermediate. The endothelium in an adult human is estimated to consist of 1×10^{13} ECs [1]. The endothelium in physiological condition is a

semipermeable layer acting as a selective barrier for the components of blood, gas, and nutriment. The EC plays a critical role in cardiovascular homeostasis regulating the vascular tone, platelet aggregation, and the adhesion of monocyte and leukocyte to the vessel wall, for this reason they are define as cell in the front-line against vascular disease [2]. In addition, the endothelium has an important role in the regulation of arterial tone as it produces both relaxant and contracting factors known as endothelium-derived relaxing factor(s) (EDRFs) and endothelium-derived vasoconstricting factor(s) (EDCFs) respectively, with a balance between EDRFs and EDCFs which varies depending on the physiological or pathological conditions. Among the EDRFs we can find for example nitric oxide (NO), prostacyclin (PGI_2), prostaglandin (PGE_2) and hydrogen sulfide (H_2S) whereas among EDCFs we can find endothelin-1 and thromboxane A2.

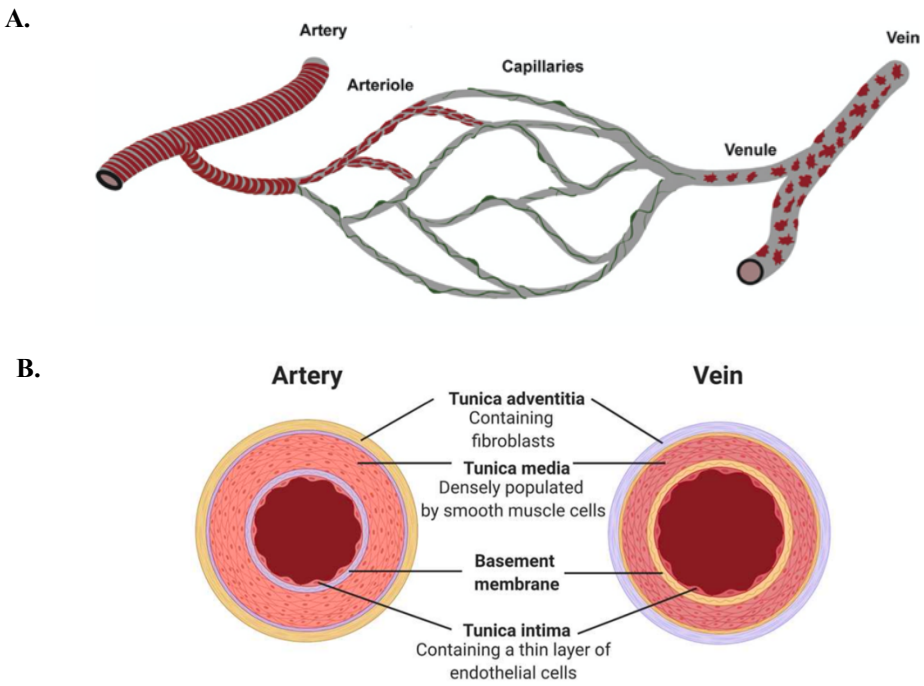


Figure 1: A. Schematic vascular branching [3] and **B.** Schematic vascular vessel components [4]

1.1 Mechanism of contraction and relaxation of vascular SMCs

The important step to initiate the contraction of SMC is the calcium (Ca^{2+}) concentration. For example, an agonist as phenylephrine (PE) binds to his membrane receptor, the α1 adrenergic receptor coupled to a G protein, and this bond actives phospholipase C that cuts the phospholipid phosphatidylinositol biphosphate (PIP_2) into two second messengers the inositol triphosphate (IP_3) and diacylglycerol (DG). IP_3 stimulates the exit of Ca^{2+} from the endoplasmic reticulum whereas the DG actives the protein kinase C (PKC) causing ROC and VOC channels opening, which are membrane channels permeable to Ca^{2+}. As we can see in figure 2, when Ca^{2+} concentration increases inside the cell cytoplasm, it interacts with calmodulin and activates the myosin light chain kinase (MLC kinase) that phosphorylate the myosin light chain (MLC) and permit the interaction of MLC and actin through the use of adénosine-5'-triphosphate (ATP), allowing in this way the contraction. This mechanism of action is also associated to 5-hydroxytryptamine (5-HT) induced vasoconstriction. Another contracting molecule is the potassium chloride (KCl) that it is used to escape the activation of the receptor coupled to G protein (GPCR); KCl activates the contraction through the intracellular increase of Ca^{2+}. As shown in figure 3, in the opposite way the vasorelaxation of SMC happens when there is a lowering of intracellular Ca^{2+} concentration and the activation of myosin light chain phosphatase (MLC phosphatase) which dephosphorylates the MLC. For example, acetylcholine (ACh) followed the binding with the muscarinic receptors in ECs caused an increase in Ca^{2+} that actives, through calmodulin, the endothelial nitric oxide synthase enzyme (eNOS) to produce NO. NO diffuse through the SMC membrane and activate guanylate cyclase that converts guanosine triphospohate (GTP) into 3',5'-cyclic guanosine monophosphate (cGMP). The increase in cGMP activates cGMP-dependent protein kinase (PKG). In this way, there is a reduction of Ca^{2+}, by a sequestration in the endoplasmic reticulum, by the extrusion of Ca^{2+} outside the SMC membrane and the reduction of influx of Ca^{2+} from the channels.

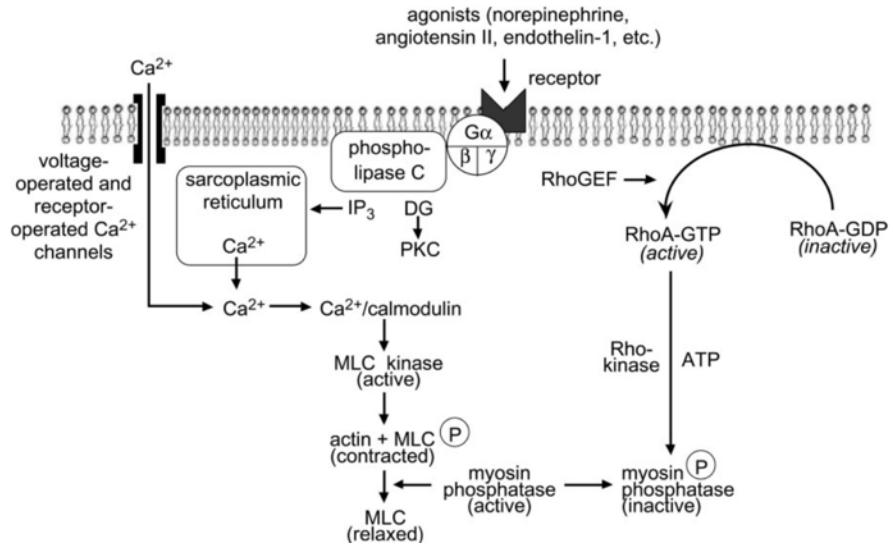


Figure 2: Regulation of SMC contraction; inositol triphosphate (IP_3), diacylglycerol (DG), myosin light chain kinase (MLC kinase) and myosin light chain (MLC) [5].

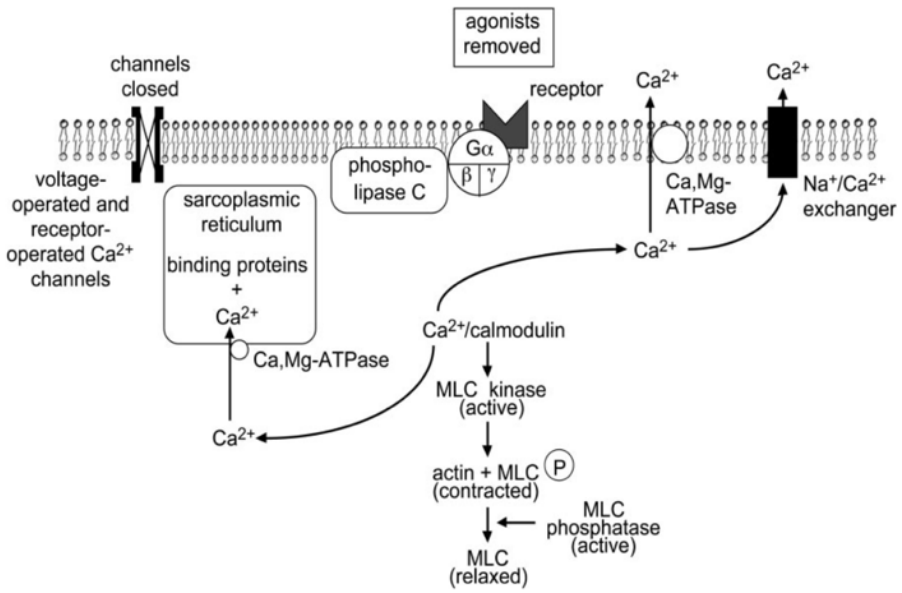


Figure 3: Regulation of SMC relaxation; myosin light chain phosphatase (MLC phosphatase), myosin light chain kinase (MLC kinase) and myosin light chain (MLC) [5].

1.2 Cardiovascular pathology and endothelial dysfunction

Cardiovascular diseases (CVDs) are the largest cause of death with 32% of all global death in 2019, representing a considerable burden to the world's health resources and increasing due to aging population and eating habits [6]. In 2013, the World Health Organization (WHO) planned a ''Global action plan for the prevention and control of noncommunicable diseases (NCD) 2013-2020'' to reduce the number of deaths for NCDs. One important point is to reduce the global prevalence of raised blood pressure and the correct identification of sign to prevent heart attack and stroke. The 26,4% of the world's adult population had arterial hypertension, this percentage is estimated to grow to 29% by 2025 [7]. Abnormality of the vascular endothelium is one of the major causes of CVDs such as atherosclerosis, arterial hypertension, coronary artery disease, ischemia reperfusion injury and myocarditis [8]. The dysfunction of ECs is characterized by disrupted vascular tone between EDRFs and ECRFs, redox unbalance, and increase inflammation, as shown in figure 4. The first definition of endothelial dysfunction was suggested by Hunt and Jurd in 1998 [9], including five characteristics:

- Loss of vascular integrity
- Increased expression of adhesion molecules
- Prothrombotic phenotype
- Production of cytokines
- Increased leukocyte adhesion to EC

It is widely demonstrated that the endothelium represents an important part of the vasculature and, by releasing several factors, it has an important role in the regulation of vascular tone. In particular, gasotransmitters are vasorelaxant agents and the endothelial dysfunction is mainly associated with a decrease availability of NO and H_2S [10].

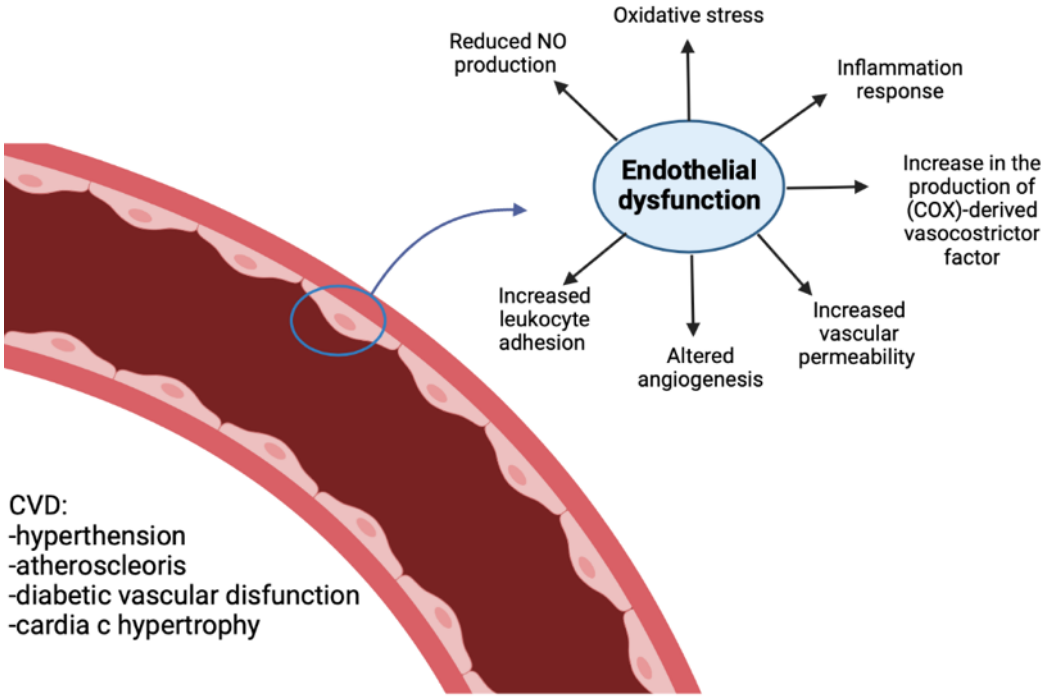


Figure 4: Characteristics of endothelial dysfunction in CVD; Cardiovascular disease (CVD) and cyclooxygenase (COX). Created with BioRender.com.

1.3 The gasotransmitters in vascular system

The gasotransmitters are a group of small gaseous molecules including NO, H_2S and carbon monoxide (CO). They are gases at standard temperature and pressure, but they are solutes in intracellular and extracellular fluids. This term was introduced in 2002 [11] to describe this group of signaling molecules characterized by a high lipid solubility that allows them to pass the membrane without a receptor and a short half-life. So, they have the ability to reach other cells in a short period of time. NO and CO were the first discovered whereas the physiological role of H_2S was discovered later. It is a class of molecules with an important role in health and disease. Interestingly, they are toxic to human at high concentrations, and play a role as signaling molecules a low concentration.

1.3.1 Nitric oxide

The NO was discovered through the observation of the vasorelaxant effect of ACh on the endothelium. This discover was done by Robert Funchgott, Ferid Murad and Luis Ignarro in 1987 (Nobel price). In the vasculature, NO is synthesized by eNOS which catalyzes the oxidation of L-arginine (L-Arg) to produce NO and L-citrulline. In total there are three identified forms of NOS:

1. the neuronal NOS (nNOS), NOS I, constitutively expressed in central and peripheral neurons
2. the inducible NOS (iNOS), NOS II, expressed in inflammatory conditions
3. the endothelial NOS eNOS, NOS III, mostly present in the ECs.

In all the forms of NOS, it is necessary to have (figure 5) [12]:
-a substrate which is L-Arg
-a co-substrate: one molecule of oxygen and a reduced nicotinamide-adenine-dinucleotide phosphate (NADPH)
-a cofactor: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R-) 5,6,7,8-tetrahydro-L-biopterin (BH_4)

All the NOS proteins are homodimer and as monomer the enzyme is unable to synthetize NO because it is unable to bind the cofactor BH_4 and the substrate L-Arg. It has to be remembered that L-Arg (substrate of NOS) is cleaved by Arginase 1 and Arginase 2 (Arg-1 and Arg-2) to form urea and L-ornithine. In cardiovascular system, the first important action of NO is the regulation of vasorelaxation but is not the only one role. It has also an atheroprotective effect, it is a potent inhibitor of platelet aggregation and adhesion and it has a role also in inflammation. In fact, a reduction of NO is associated to an increased expression of adhesion molecule as vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) and so there is an augmentation of platelet aggregation and leukocyte adhesion [13]. Thus, the NO can also suppress the adhesion of leucocytes to ECs.

More in details, ECs in some condition as inflammatory stimuli are able to express in the surface some adhesion molecule that facilitate the adhesion of leucocytes. So, the first step is the exposure cell adhesion molecule on the surfaces of activated ECs, as ICAM and VCAM. As the nNOS, for the activation of eNOS the presence of Ca^{2+} and calmodulin are essential as the presence of Ca^{2+} allows the binding of calmodulin on the eNOS. On the contrary, iNOS is Ca^{2+} independent and it is expressed in presence of infection and inflammation [14]. There are also other differences between the eNOS and nNOS with the iNOS; for example, the first two produce low concentration of NO (nM) in a short framework whereas iNOS produce high concentration of NO (μM) with a long duration. Besides calmodulin, heat shock protein 90 (hsp90) is another protein important for the activity of eNOS. In the inactive conformation eNOS bounds to caveolin-1 (Cav-1), as we can see in figure 6, and when there is the recruitment of hsp90 and calmodulin, the eNOS leaves Cav-1 and becomes active to synthetize NO. Moreover, the eNOS can be phosphorylated (peNOS) on serine, threonine, or tyrosine and depending on the site of phosphorylation could lead an activation or inhibition of the activity. The most important phosphorylation is on serine1177 that activates the synthesis of NO. Other sites of phosphorylation are the serine615 and the serine635. The site of inhibition by phosphorylation is the threonine495. Among the activators of eNOS, ACh allows an increase of Ca^{2+} with the activation of calmodulin and thus the eNOS can synthetize NO. More specifically, when ACh binds to his GPCR in EC, it activates phospholipase C and produces IP_3 that cause an increase in Ca^{2+} concentration; Ca^{2+} binds to calmodulin activating eNOS to produce NO. As described before, after its release from the ECs, NO diffuses in the vascular SMCs, increases cGMP production and leads to the activation of the PKG causing relaxation through a decrease in intracellular Ca^{2+} concentration. As NO has an important vasorelaxant effect, deletion of the eNOS gene induces an elevation in blood pressure [15]. When NO interacts with superoxide anion ($O_2^{-\bullet}$) it forms peroxynitrite ($ONOO^-$) that can induce the nitration and oxidation of proteins, including eNOS, and this can reduce the

production of NO leading to arterial hypertension [16]. Moreover, $ONOO^-$ can also oxidate the BH_4 to form 7,8-dihydrobiopterin (BH_2) reducing in this way the cofactor important to the NOS activity. Thus, the reaction between NO and $O_2^{\bullet-}$ limits NO availability and results in eNOS uncoupling, with an augmentation of oxidative stress that contribute to the endothelial dysfunction onset. In addition, in a clinical study it has been demonstrated that a genetic predisposition to enhanced NO signaling is linked to reduced level of blood pressure [17].

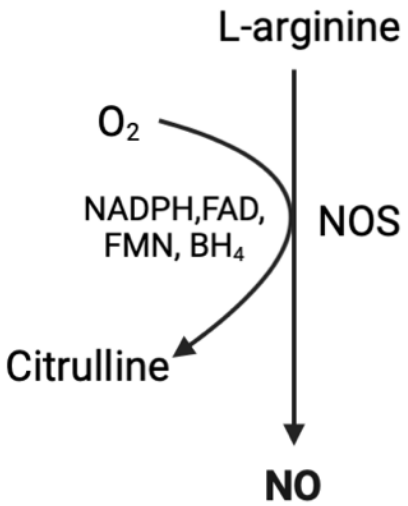


Figure 5: NO synthesis; nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), (6R-) 5,6,7,8-tetrahydro-L-biopterin (BH_4), nitric oxide synthase enzyme (eNOS) and nitric oxide (NO). Created with BioRender.com.

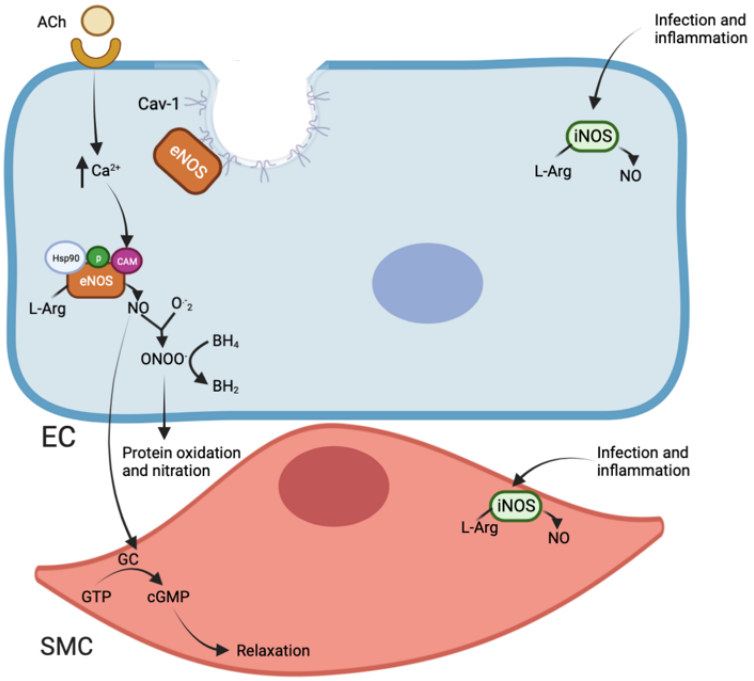


Figure 6: Regulation of eNOS and iNOS activity; nitric oxide synthase enzyme (eNOS), nitric oxide (NO), caveolin-1 (Cav-1), peroxynitrite ($ONOO^-$), (6R-) 5,6,7,8-tetrahydro-L-biopterin (BH_4), 7,8-dihydrobiopterin (BH_2) guanosine triphospohate (GTP) into 3',5'-cyclic guanosine monophosphate (cGMP). inducible NOS (iNOS), acetylcholine (ACh), endothelial cell (EC) and smooth muscle cell (SMC). Created with BioRender.com.

1.3.2 Hydrogen sulfide

H_2S is the last gasotransmitter discovered and in the last decade its role in the pathogenesis of vascular dysfunction has grown exponentially. In 1942 Vincent du Vigneaud has demonstrated the production of H_2S in mammalian tissue homogenates. Nevertheless, it was considered as a biological mediator by Abe and Kimura only in 1996. They demonstrated the ability of H_2S to act as a neuromodulator, opening in this way a new field in life science [18]. As show in figure 7, H_2S is synthesized by three enzymes, constitutively expressed; cystathionine-beta-synthase (CBS), cystathionine-gamma-synthase (CSE), using as substrate L-cysteine (L-Cys) and homocysteine, and the third 3-mercaptopyruvate sulfur transferase (3-MST) that acts in tandem with cysteine aminotransferase (CAT) providing the substrate 3-mercaptopyruvate for 3-MST. The first two enzymes are cytosolic whereas 3-MST and CAT are localized in both the cytosol and mitochondria. Differently from the eNOS, all the enzymes involved in H_2S biosynthesis are present within the vasculature, in both SMCs and ECs [19, 20, 21]. CSE is the most important enzyme present in the vasculature and this is supported by the observation that $CSE^{-/-}$ mice develop arterial hypertension [22]. In parallel, patients with arterial hypertension, show plasma levels of H_2S lower than normotensive subjects [23]. Also, in a rat model of hypertension, a donor of H_2S as sodium hydrosulfide (NaHS) lowers the blood pressure [24]. More specifically the circulating level of H_2S is a predictor of the development of arterial hypertension [25]. So, the exogenous administration of H_2S donor as sodium sulfide (Na_2S) and NaSH can reduce the blood pressure in an animal model of hypertension [26]. The molecular targets of H_2S in the SMCs are the ATP-dependent potassium (K_{ATP}) [27], L-type Ca^{2+} [28], large-conductance Ca^{2+} activated potassium [29], and voltage-dependent potassium channels [30], all inducing relaxation. Furthermore, H_2S inhibits phosphodiesterase activity, due to its sulfidation, the enzyme that catabolizes cGMP and 3',5'-cyclic adenosine monophosphate [31]. It has also been reported that the activation of phospholipase A2 is another target of H_2S [32]. Moreover, H_2S has

an important antioxidant role as it blocks oxidative modification of low-density lipoprotein, reducing the atherosclerosis lesion [33], and also through the elimination of oxidative hemoglobin [34]. Finally, it has also been demonstrated that the up-regulation of CSE/H_2S pathway could attenuate the atherosclerosis [35].

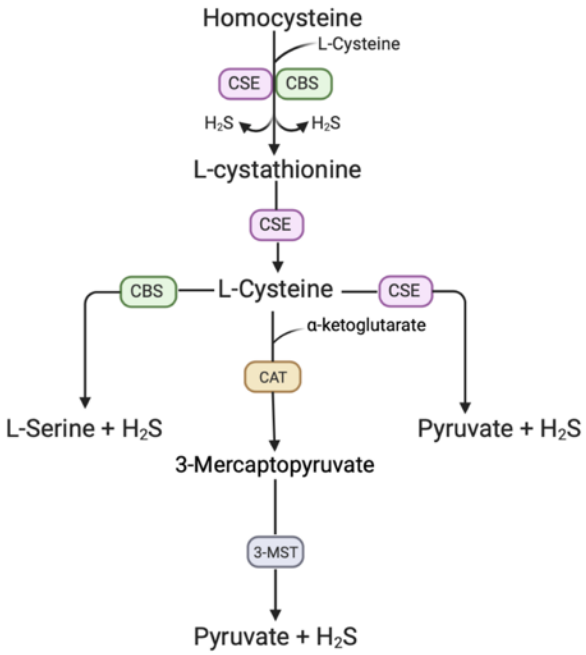


Figure 7: Biosynthesis of H_2S; cystathionine-beta-synthase (CBS), cystathionine-gamma-synthase (CSE), 3-mercaptopyruvate sulfur transferase (3-MST), cysteine aminotransferase (CAT) and hydrogen sulfide (H_2S) [36].

1.4 NO and H_2S in the vascular system

As reported before, a reduced NO and H_2S bioavailability is associated with CVD, especially in the pathogenesis of arterial hypertension. Thus, they play a key role to regulate vascular homeostasis. NO function primary through the guanylate cyclase/cGMP and H_2S through K_{ATP} channel and, as reported before, both lead to the relaxation of vascular SMCs. Moreover, it is important to remember that when there is an endothelium dysfunction NO and H_2S decreases but H_2S can be produced also from the SMC; this can offer an advantage for H_2S contribution in the control

of vascular tone. Intriguingly, these two gasotransmitter regulate each other and they are mutually dependent in the regulation of endothelium-dependent relaxation. For example, H_2S can increase the NO activity by a directly action or through Akt activation of eNOS [37] (figure 8), and this effect does not apply iNOS or nNOS. On the other hand, eNOS ablation impairs H_2S-induced relaxation and in the same way, the NO donor-dependent vasorelaxation is attenuated in absence of CSE [38]. H_2S is also able to stimulate the eNOS activity by its persulfidation [39]. Recently an endogenous cross-talk between NO and H_2S has been demonstrated in which CBS plays a key role [40].

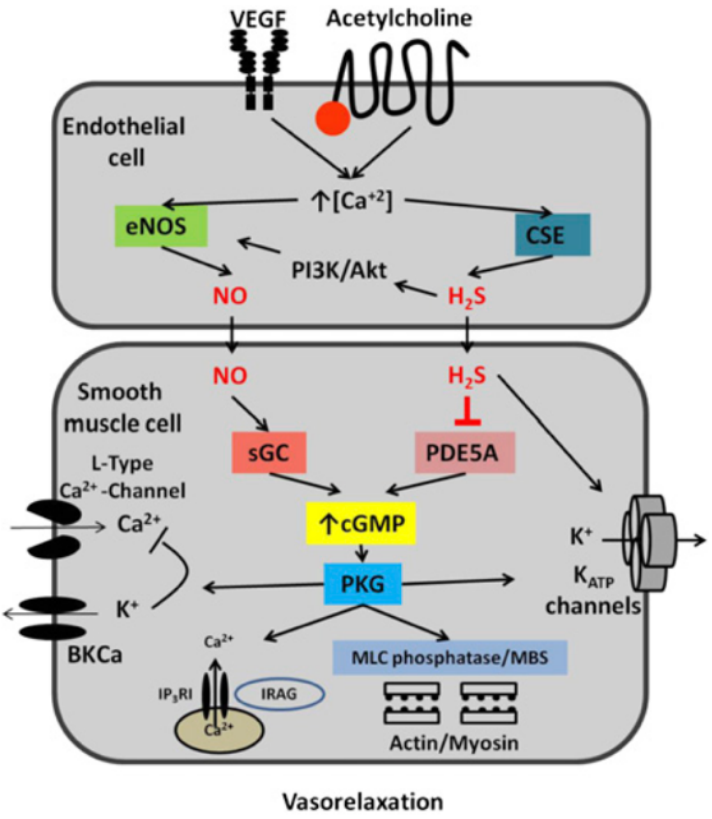


Figure 8: Interaction between H_2S and NO; phosphodiesterase (PDE), cystathionine-gamma-synthase (CSE), guanylate cyclase (GC), nitric oxide synthase enzyme (eNOS) and nitric oxide (NO) and myosin light chain (MLC) [41].

CHAPTER II
GLUCOCORTICOID INDUCED LEUCINE ZIPPER PEPTIDE AND VASCULATURE

2.1 Glucocorticoids

Synthetic glucocorticoids (GCs) could be described as a 'live-saving' drug for its important action in several inflammatory and autoimmune diseases. The importance of the glucocorticoid is so fundamental that in 1950 the Nobel Prize was given to the discoverers Edward Calvin Kendall, Philip Showalter Hench and Tadeus Reichstein. GCs are widely used for autoimmune disorder, chronic inflammation, allergic reaction and organ transplants. Cortisol is the main natural glucocorticoid and it has various biological effects as the regulation of the metabolism, cardiovascular function, a role in growth and in the development of immune response. The mechanism of action of GC is complex; GCs move inside the cells and bind to the glucocorticoid cytoplasmatic receptor (GR). The complex receptor-ligand moves into the nucleus and binds to the glucocorticoid responsive elements (GRE) a specific DNA sequence that regulates a specific gene. The GC inhibits the transcription of pro-inflammatory and immunostimulant proteins like cytokines, chemokines, growth factor, adhesion molecules and enzymes, in the other hand they bring the induction of anti-inflammatory protein like annexin-1, IKBα, Glucocorticoid Induced Leucine Zipper (GILZ) and MAPK phosphatase-1. We can distinguish the transrepression action of GC which is the repression of inflammatory transcription factor and the transactivation action of GC that is related to the physiological metabolic action and side effect, as we can see in the figure 9. The chronic use of the GCs is accompanied by numerous side effects for example arterial hypertension, hyperglycemia, osteoporosis and Cushing's syndrome [42]. The GCs's hypertension could be related to a receptor mediated mechanism, since a potent glucocorticoid antagonist RU 486 prevents glucocorticoid-induced hypertension [43]. The GC hypertension could in part be due to the renal sodium retention [44], but on the other hand spironolactone, specific antagonist of aldosterone (hormone that control the

balance of water and salts in kidney) receptor, is unable to prevent this kind of hypertension [45]. Another mechanism of the GC induced hypertension is the increase of norepinephrine and angiotensin II (Ang II) effect in inducing a higher-pressure response [46] and also the Ca^{2+} influx [47]. It has been demonstrated that GR has a direct effect on hypertension since these receptors are also expressed on endothelial and vascular smooth muscle cells; the suppression of the GRs in vascular smooth muscle doesn't prevent the hypertension [48] while interestingly, the deletion of GR in ECs completely annuls this effect [49]. Thus, it's necessary to exploit new therapeutic approaches and/or ameliorate those that are already in use to prevent, avoid or at least reduce the related side effect.

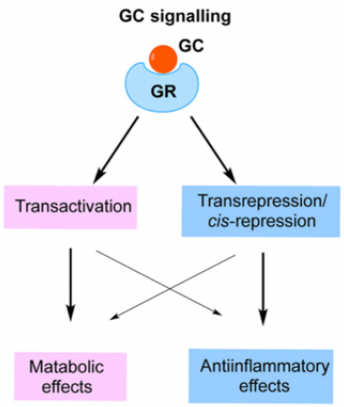


Figure 9: GC transactivation and transrepression; glucocorticoids (GCs) and glucocorticoid receptor (GR) [50].

2.2 Glucocorticoids in vasculature

A reduction of NO and H_2S is also involved in the side effects associated to systemic and chronic administration of GCs, which is arterial hypertension. In fact, *Wallerath et al.* have demonstrated that GCs bring a down regulation of eNOS [51] and that dexamethasone (DEX), a synthetic GC, increases the arterial blood pressure in wild type (WT) mice but not in the $eNOS^{-/-}$ mice [52]. Moreover, glucocorticoid decreases

the expression of GTP cyclohydrolase (GTP-CH) and the level of BH_4, reducing the activity of eNOS [53]. Thus, the reduction of eNOS induced by DEX could contributes to the arterial hypertension caused by glucocorticoid. In addition, DEX acts at different levels since it causes down regulation of cationic amino acid transporter-1 (CAT-1), which allow the entrance of L-Arg, and increasing the generation of reactive oxygen species (ROS) [54, 55]. Moreover, DEX can also stimulate the Cav-1 expression, inhibiting in this way the eNOS activity [56]. As well as, DEX treatment leads to a reduction of H_2S generation in carotid and mesenteric beds in rats [57]. The results show a significant reduction in vascular districts of the expression of CBS and CSE, enzymes involved in the production of the H_2S from L-Cys. This reduction was confirmed by measuring the levels of H_2S in the plasma [58]. In conclusion, as showed in figure 10, DEX is able to inhibits both synthesis of NO and H_2S showing its detrimental role in vascular diseases.

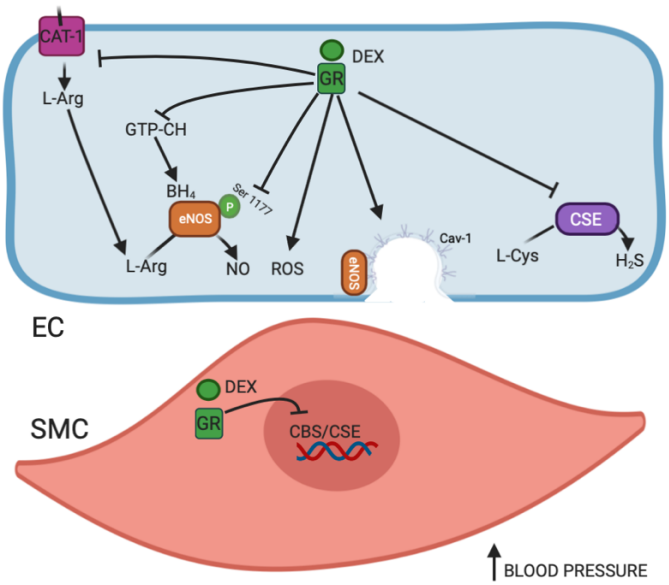


Figure 10: DEX effect on NO and H_2S synthesis; dexamethasone (DEX), glucocorticoid receptor (GR), cystathionine-gamma-synthase (CSE), hydrogen sulfide (H_2S) nitric oxide (NO), caveolin-1 (Cav-1), L-arginine (L-Arg), cationic amino acid transporter-1 (CAT-1), of GTP cyclohydrolase (GTP-CH), endothelial cell (EC) and smooth muscle cell (SMC). ⟶ : stimulation; ⟞ inhibition. Created with BioRender.com.

2.3 Glucocorticoid Induced Leucine Zipper

Between the strategy for separating therapeutic from adverse effects of GCs there is the use of a new ligand: GILZ. GILZ is a protein that was first discovered in 1997 by *D'Adamio* [59] and it is identified as a protein induced by DEX, and acts by miming the action of GCs. Other studies have shown that more than three GREs exist in the region of GILZ transcriptional start [60]. Human GILZ is a 135-aa protein, ubiquitously expressed, formed by three domains [61]: N-terminal domain, a central leucine zipper domain, important for its homodimerization and C-terminal domain, which has a proline/glutamate-rich region which interacts with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (figure 11A). GILZ directly interacts with p65 and p52 subunits of NF-kB [62] (figure 11B), inhibiting its nuclear translocation and DNA binding. NF-kB is an important transcription factor essential for the modulation of inflammatory and immune responses, to clarify this point it has been proposed that GC induced the up-regulation of GILZ that in turn control the activation of NF-kB [63]. Moreover, the anti-inflammatory effect of GILZ on ECs involves the inhibition of NF-kB [64]. In addition, they find that that GILZ is substantial express in ECs and that in tumor necrosis factor (TNF-α) treatment the GILZ expression is downregulated, and absence of GILZ enhanced NF-kB activity in ECs [64]. In addition, it has also demonstrated that in human umbilical vein EC (HUVEC) the overexpression of GILZ bring a reduction of inflammatory response due to TNF-α [65]. According to this, in retinal microvascular EC, it has also demonstrated that in GILZ overexpression inhibits the inflammatory response after lipopolysaccharide (LPS) treatment [66]. Many experimental models support the possibility of the use of GILZ instead of GC. The injection of a GILZ fusion protein, TAT-GILZ, shows a reduction of colitis [67] and the injection of GILZ-P, a proline-rich segment, lead to an efficient treatment of encephalomyelitis [68]. It is known that transgenic mice that over express GILZ (TG-GILZ) are more resistant while GILZ knockout (KO) ($GILZ^{-/-}$) mice develop worse inflammatory diseases. Some results suggest that a synthetic GILZ–derived

peptide, could be the basis for designing new therapeutic approaches to inflammatory diseases. These results support the hypothesis of using GILZ pathway like a base for designing new anti-inflammatory drugs.

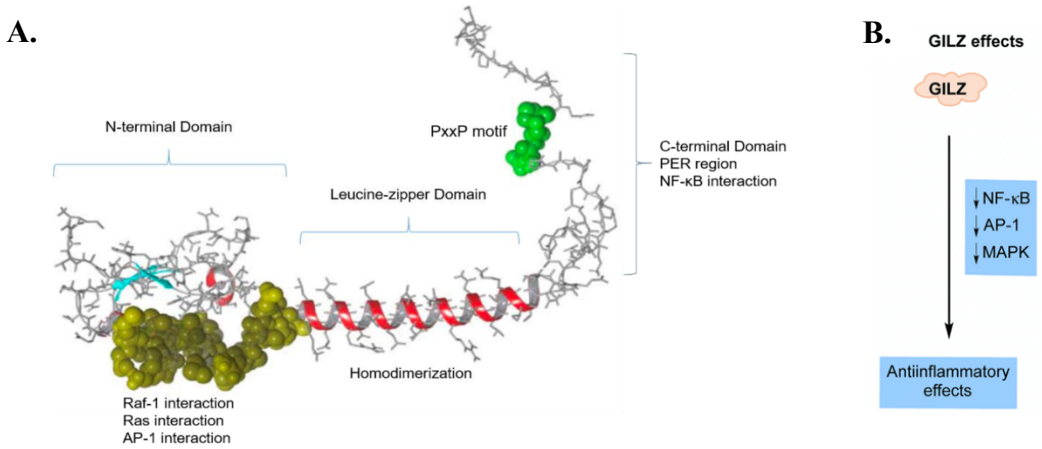


Figure 11: A. Structure of Glucocorticoid Induced Leucine Zipper (GILZ) [61] **B.** GILZ effect; and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [50]

2.4 Aim

As stated before, the chronic use of the GCs is accompanied by second effects some of those impact on the cardiovascular system as for example the arterial hypertension. It has been found that GILZ is substantial express in ECs and that the downregulation of GILZ could bring atherogenesis [63]. As we know NO and H_2S have antiatherogenic effect. Thus, to assess the possibility to use GILZ in alternative to GCs, avoiding their side effect of hypertension development, we evaluate the contribution of NO and H_2S signalling in $GILZ^{-/-}$ mice.

2.5 Materials and methods

2.5.1 Animals

GILZ$^{-/-}$ male mice and their WT littermates the C57BL/6J were used. GILZ$^{-/-}$ were generated as previously described, mice containing a floxed GILZ allele were generated on C57BL/6J mice [69] and they were provided by the research group of prof Carlo Riccardi (University of Perugia). They were housed in the animal care facility at the Department of Pharmacy, University of Naples, Italy, in a kept under environment temperature (21 ± 2°C) and humidity (60 ± 10%), and 12-h light/dark cycles. Food and water were fed *ad libitum* and provided with free access to standard rodent chow and water. All animals were allowed to acclimate at least 5 days before experiments and were subjected to a 12-hr light-dark schedule. Experiments were conducted during the light phase. Mice were randomly used for ex vivo experiments. All effort was taken to minimize the number of animals used and their suffering. The present study was approved by the Animal Ethics Committee of the University of Naples "Federico II" (Italy), in agreement with both Italian and European guidelines for animal care.

2.5.2 Tissues and plasma preparation

Mice were euthanized by overdose of enflurane. The blood was collected by cardiac puncture and mixed with trisodium citrate (3.8%, wt/vol; Sigma). Plasma was obtained by centrifugation 13000 rpm for 15 min at room temperature. The aorta was harvested and cleaned from connective and fat tissue. Tissue was placed in dish containing oxygenated Krebs' solution (NaCl 118 mM, KCl 4.7 mM, $MgCl_2$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, and glucose 10.1 mM; Carlo Erba Reagents S.r.l., Milan, Italy) kept at 37°C and then used for ex vivo vascular reactivity and for H_2S or NOx determination.

2.5.3 Ex vivo vascular reactivity

Aortas harvested from both GILZ$^{-/-}$ and WT were cut in rings of 1–1.5 mm length, mounted in isolated organ bath (3.0 mL) filled with oxygenated (95% O_2–5% CO_2) Krebs' solution and connected to an isometric force transducer (FORT10, 2Biological Instruments, Varese, Italy). The changes in tension were continuously recorded by a computerized Power Lab System (MacLab System, ADInstruments software). Rings were stretched to a resting tension of 1.5 g and allowed to equilibrate for 30 minutes. In each set of experiments, rings were firstly challenged with PE (1 μM, Sigma-Aldrich, Milan, Italy) until the responses were reproducible. In order to see if there were any change in contraction effect, we perform a cumulative concentration-response curve (CCRC) to PE (1 nM–30 μM, Sigma-Aldrich), 5-HT (1 nM–30 μM, Sigma-Aldrich) and KCl (1 mM–0.1 M, Sigma-Aldrich). The relative NO contribution in relaxation was verified by performing a CCRC to ACh (0.01–30 μM, Sigma-Aldrich) on PE (1 μM) stable tone. L-NG-nitro-arginine methyl ester (L-NAME, non-specific NOS inhibitor; 100 μM, Sigma-Aldrich) was added on stable tone of PE (1 μM), to see if there were any change in the basal eNOS activity. In another set of experiments, in order to see if GILZ peptide could stimulated the activity of eNOS, we used the lyophilized GILZ peptide (PROTEOGENIX) alone or in combination with L-NAME (100 μM). The intracellular delivery of GILZ peptide was achieved by using the cell-penetrating peptide TAT (YGRKKRRQRRR) to the amino terminus of the GILZ peptide (YGRKKRRQRRRKTLASPEQLEKFQSRLSPEEPAPEAPETPEAPGGSAV) [70]. To this aim, it was performed a CCRC to PE (1 nM–3 μM, Sigma-Aldrich) followed by incubation with vehicle (H_2O) or GILZ peptide (1 μM) for 30 minutes. Thereafter we performed another CCRC to PE after incubation with vehicle + L-NAME or GILZ peptide (1 μM) + L-NAME (100 μM) for 30 minutes. In a separate set of experiments, in order to evaluate the H_2S contribution, a CCRC with L-Cys, (0.01–3000 μM, Sigma- Aldrich), the substrate of H_2S synthesized enzymes, and of NaHS (0.01–3000 μM, Sigma- Aldrich), H_2S donor, was performed in PE

precontracted rings. Data were reported as % of relaxation respect to PE tone or as increase in contraction (dyne/mg tissue). Data were expressed as mean ± standard error of the mean (SEM). Statistical evaluation was performed by using two-way ANOVA followed by Bonferroni as post hoc test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

2.5.4 NOx determination

Total nitrate content (NOx) that encloses anion nitrate (NO_3^-) and nitrite (NO_2^-), was evaluated in plasma, in aorta and in mesenteric arteries homogenates, harvested from GILZ$^{-/-}$ and WT mice. Vascular tissues were homogenized in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Igepal), protease inhibitor cocktail (Sigma-Aldrich) and sodium orthovanadate (10 mM). Protein concentration was determined by Bradford assay using bovine serum albumin as standard (Bio-Rad Laboratories, Milan, Italy). Later on, tissue homogenates or plasma were incubated with STB (3 parts of NH_4Cl 0,49M and 1 part of $Na_2B_4O_7$ 0,06M) in a microplate with cadmium (Sigma-Aldrich; 50 mg/well) for 1 hr in order to convert the inorganic anion NO_3^- to NO_2^- and then centrifuged at 12000 rpm at 4°C for 15 minutes [39]. We add the 2,3 diaminonaphtalene (DAN) which reacts with NO_2^- and gives the formation of the fluorescent product, 1-(H)-naphthotriazole. The reaction was terminated with the addition of NaOH 2.8 N. NOx was fluorometrically determined in microtiter plates by Promega Glomax explorer (Madison, WI) and calculated against a standard curve of sodium nitrite ($NaNO_2$, 50–2000 nM; Sigma-Aldrich). Data were reported as nmol per mg of protein (for the aorta and mesentery) and as nM (for plasma) and expressed as mean ± SEM. Statistical evaluation was performed by using Student t test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

2.5.5 H_2S determination

H_2S production was assessed as previously described [71]. The aortic and mesenteric arteries were homogenized in potassium phosphate buffer. The homogenates were added to a reaction mixture (total volume 500 µl) containing piridoxal-5-phosphate (2 mM, 20 µl, Sigma-Aldrich) and in case of stimulated sample was added L-Cys (10 mM Sigma-Aldrich). Trichloroacetic acid solution (10% wt/vol, Carlo Erba Reagents) was added to each sample followed by zinc acetate (1% wt/vol Sigma-Aldrich). Next, N,N-dimethyl-p-phenylendiamine sulfate (20 mM, Sigma-Aldrich) and $FeCl_3$ (30 mM, Sigma-Aldrich) were added, and optical absorbance of the solutions was measured at a wavelength of 668 nm. All samples were assayed in duplicate, and H_2S concentration was calculated against a calibration curve of NaHS (3.9–250 mM). Data are reported as nmol per mg of protein per minutes (for the aorta and for mesentery) and as nM (for plasma) and expressed as mean ± SEM. Statistical evaluation was performed by using t test by using GraphPad Instant (Graphpad Software Inc., San Diego, CA).

2.5.6 Analysis of protein expression levels by western blot

Aorta harvested from $GILZ^{-/-}$ and WT was homogenized and determined as described for NOx determination. For Western blot analysis, tissue lysate was dissolved in Laemmli's sample buffer, boiled for 5 min. Equal amounts of denatured proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) at 90 V and transferred to nitrocellulose membrane at 250 mA for 45min at room temperature. The filter was then blocked with 1x Phosphate-buffered saline (PBS), 5% non-fat dried milk and NaF (50 mM) for 40 min at room temperature and probed with CSE (1:1000; Santa Cruz Biotechnology, Inc.), eNOS (1:1000; BD biosciences), Cav-1 (1:1000; Elabsciences) and Arg-1 (1:500; Cell Signaling) dissolved in 1x PBS, 5% non-fat dried milk, 0.1% Tween 20 at 4°C, overnight. After,

the membranes were washed for four times with 1x PBS containing 0.1% Tween 20 and 5% non-fat dried milk and subsequently probed for 2h at room temperature with anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugate (1:3000). The filters were then washed in 1x PBS containing 0.1% Tween-20 for four times. Membranes were detected with enhanced chemiluminescence (ECL) immunoblotting detection reagents. Bands were quantified using a Chemi Doc (Bio-Rad) chemiluminescent detection system and densitometrical analysed using ImageQuant-400 (GE Healthcare, USA). The target protein band intensity was normalized over the intensity of the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000, Sigm-Aldrich). Data are expressed as mean ± SEM. Statistical evaluation was performed by using t test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

2.6 Results

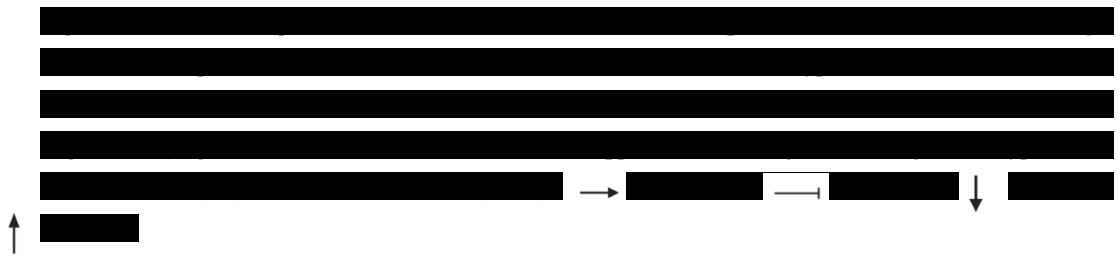
[redacted]


eNOS (14
KDa)
GAPDH (3
KDa)

[redacted]

[redacted]

[redacted]



CHAPTER III
DEHYDROEPIANDROSTERONE EFFECT IN VASCULATURE

3.1 Role of sex hormones in vasculature through NO and H_2S

As we known sex hormones varies during the development and the age, and the role in the control of vascular homeostasis is widely demonstrated [75]. Sex hormones influence the synthesis of the two gasotransmitters NO and H_2S and thus they influence the cardiovascular homeostasis (figure 20). Testosterone (T), the male sex hormone stimulates both NO and H_2S. In fact, in physiological concentration, T induces an endothelial-dependent relaxation. In contrast, a higher pharmacological concentration of T produces an endothelial independent relaxation. *More in details,* physiological (10^{-9}/10^{-8}) concentration of T in vitro activates NO synthesis by phosphorylation of eNOS extracellular signal-regulated kinases *(*ERK1/2) and PI3K/Akt. It has been also shown an augmentation in eNOS expression. This effect was only observed at a physiological concentration of T and in a higher concentration don't bring any augmentation of eNOS expression [76]. In contrast, T at higher concentration *(*(10^{-7}/10^{-4}) induces a vasorelaxation in a non-genomical and endothelial independent way. This endothelium independent effect seems to involve the activity of different channels; T inhibit L-type Ca^{2+} channels and activates potassium channels in SMC [77]. In this action also takes part H_2S; T is a positive modulator of the CSE activity but not on their expression. In fact, no change of the expression of the enzyme that synthesized H_2S after T treatment has been observed. Moreover, an augmentation of the H_2S levels after T treatment of the aorta has been observed. Indeed, T-induced vasodilatation also involved K_{ATP} channel, a known target of the H_2S vasorelaxant effect [78]. In this context, *Brancaleone et al.* have shown that the effect of T on H_2S involved a ternary complex; androgen receptor (AR) NR3C4, hsp90, and CSE, which led to the activation of the enzyme and so the synthesis of H_2S [79] (figure 20). In fact, they demonstrated a strong interaction between hsp90 and CSE, thus the activation of CSE by T happen through the interaction with hsp90. This was demonstrated by using Nilutamide (Nil) an AR

antagonist and by geldanamycin a specific hsp90 inhibitor. As regarded to the estrogen (E_2), we known that E_2 activate the eNOS enzyme by an increase of Ca^{2+} leading to its activation after phosphorylation [80]. Moreover, it acts also through H_2S. In fact, the antiatherogenic effect of E_2 involves CSE enzyme [81]. A functional study of femoral arteries has shown that H_2S has a role in E_2-dependent vasorelaxation of ACh in a non genomical way with an activation of CSE [82]. Not only E_2 has a role on the production of H_2S but also *vice versa*; CSE, probably through the action of its metabolic substrate, has a role on the production of E_2 as demonstrated by a reduction of E_2 levels observed in CSE-KO female mice [81]. Finally, the non-genomic mechanism was better clarified, E_2 activates CSE through the phosphorylation of the enzyme by PKG-I beta. E_2 interact with the membrane estrogen receptor (ERα), that interact with protein Gα that active pGC-A to produce cGMP that subsequently activates CSE [83], as showed in figure 21.

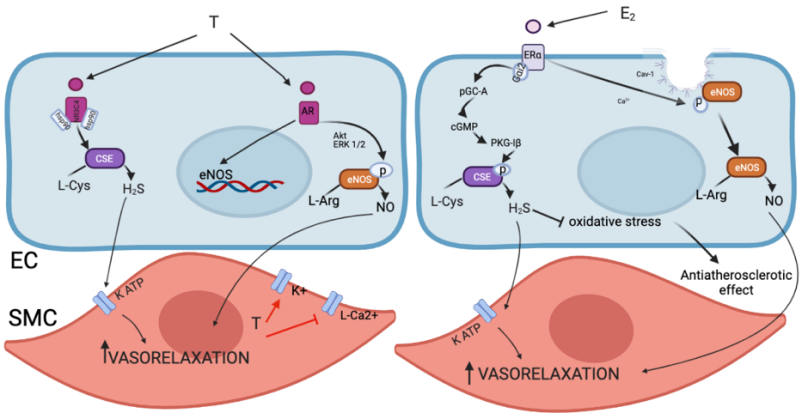


Figure 21: Testosterone and estrogen activation of NO and H_2S; cystathionine-gamma-synthase (CSE), hydrogen sulfide (H_2S), L-cysteine (L-Cys), nitric oxide (NO), caveolin-1 (Cav-1), L-arginine (L-Arg), androgen receptor (AR), estrogen receptor (ERα), endothelial cell (EC) and smooth muscle cell (SMC).

→ : stimulation; ⊣ : inhibition. Created with BioRender.com.

3.2 Dehydroepiandrosterone

Dehydroepiandrosterone (DHEA) is the most abundant circulating steroid hormone in humans and it is present in serum mainly as sulfate form ester (DHEA-S). It is a precursor of sex hormones (figure 22) and it is produced in the zona reticularis of the adrenal gland from cholesterol through the formation of pregnenolone. Its levels increase during puberty and decline with age; moreover, a lower level of DHEA have been linked to the development of vascular diseases [84] and its exogenous administration was defined as a ''fountain of youth''. In fact, DHEA has an antidiabetic, anticancer, anti-allergic, obesity treatment and cardiovascular property. Moreover, it was found that elevated levels of DHEA-S were associated with lower mortality rates from cardiovascular and other diseases [85].

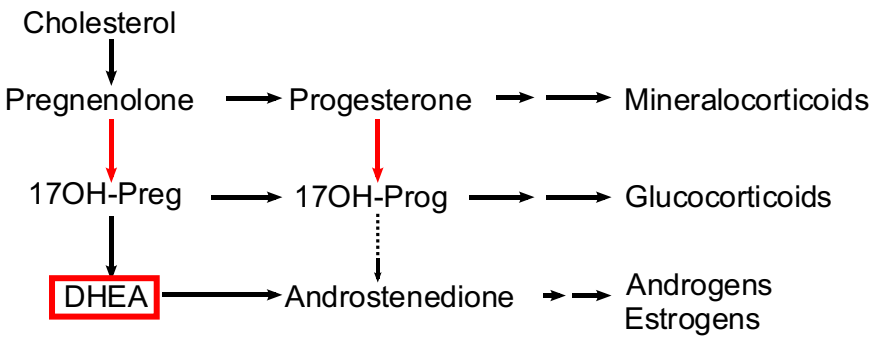


Figure 22: Steroid hormone synthesis; dehydroepiandrosterone (DHEA); *(Wikimedia Commons).*

3.3 Dehydroepiandrosterone and NO

The beneficial effect of DHEA in cardiovascular function is still addressed. In 1992 has been demonstrated that DHEA treatment in vivo prevents the GC-mediated arterial hypertension in a dose-dependent manner, but with no effect on DOCA-salt induced hypertension. In a physiological condition of stress, DHEA could prevent some deleterious effects of GC [86]. Several data have demonstrated that the beneficial effect of DHEA is associated to an increase in NO synthesis. DHEA binds to its specific membrane receptor, a GPCR, activating the eNOS independently from

estrogen and androgen receptors in human ECs. This action involves the ERK1/2 MAKC function [87]. In addition, it was found that DHEA phosphorylates the ser1177 eNOS in bovine aorta endothelial cells (BAEC) [88]. *Lu et al.* confirm the role of DHEA on the activation of eNOS by a GPCR (figure 23) [89]. Moreover, DHEA has an intrinsic antioxidant activity. For example, in ovariectomized (OVX) rats, DHEA inhibits ROS formation and restores the activation of eNOS [90]. Indeed, *Akishita et al*. demonstrate that the low plasma DHEA levels were correlated to endothelial dysfunction in postmenopausal women, showing that DHEA has a role in the development of cardiovascular diseases in postmenopausal woman [91]. In vivo studies have shown that DHEA enhances large and small vessel ECs function in postmenopausal women, and this could corroborate its potential action to ameliorated cardiovascular diseases [92]. In addition, low levels of this precursor have been linked with other pathologies such as diabetes type 2 and osteoporosis [93]. Furthermore, DHEA was negatively associated with arterial hypertension while the ratio cortisol:DHEA was positively associated [94]. In addition, DHEA treatment protects from pressure overload in aorta tissue of OVX rats through sigm-1 receptor and its activity involved AKT eNOS signaling [95]. In vitro experimentation on pig's coronary artery have shown a role of K_{ATP} activation after a stimulation with DHEA. This relaxing action was not dependent on the estrogen receptor and partially dependent on androgen receptor [96].

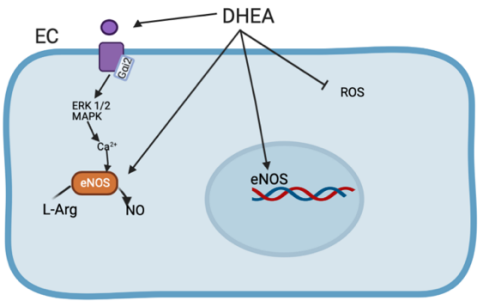


Figure 23: DHEA activation of NO; Dehydroepiandrosterone (DHEA), nitric oxide (NO), L-arginine (L-Arg), nitric oxide synthase enzyme (eNOS) and endothelial cell (EC). → : stimulation; ⊣ : inhibition. Created with BioRender.com.

3.4 Aim

As we know DHEA is a precursor of testosterone and estrogen. The aim of the present study was to clarify the vascular role of DHEA and the underlined mechanism(s). In particular, the involvement of H_2S/NO pathways in the DHEA induced relaxation has been investigated by using functional and cell cultures experimental models, with particular regard to H_2S since, no data are available concerning the potential role of DHEA on H_2S pathway.

3.5 Materials and methods

3.5.1 Animals

CD1 male mice were used in the same condition reported in 2.5.1

3.5.2 Tissue preparation

Mice were euthanized by overdose of enflurane. The aorta was harvested and cleaned from connective and fat tissue. Tissue was placed in dish containing oxygenated Krebs' solution, kept at 37°C and then used for ex vivo vascular reactivity and for H_2S determination.

3.5.3 Ex vivo vascular reactivity

Aortas harvested from CD1 mice were cut into 1–1.5 mm long ring and mounted in organ bath (3.0 mL) filled with oxygenated (95% O_2–5% CO_2) Krebs' solution and connected to an isometric force transducer (FORT10, 2Biological Instruments, Varese, Italy). The changes in tension were continuously recorded by a computerized Power Lab System (MacLab System, ADInstruments software). Rings were stretched to a resting tension of 1.5 g and allowed to equilibrate for 30 minutes. In each set of experiments, rings were firstly challenged with PE (Sigma-Aldrich) until the responses were reproducible. In order to assess the integrity of the endothelium we perform a CCRC to ACh (0.01–30 μM, Sigma-Aldrich) on PE (1 μM) stable tone. Arterial rings were considered as ‘’with endothelium’’ when the relaxation to ACh

was larger than 70%. In a separate set of experiments, we removed the endothelium. After a stable tone to PE (1 μM) we performed a CCRC to DHEA (0.1-300 μM Sigma-Aldrich). We evaluated also the relaxation effect of DHEA before and after SCH 202676 an allosteric modulator of the GPCR (1 μM, Tocris) for 40 minutes. In order to evaluate H_2S contribution in DHEA relaxation, aspartic acid (1 mM, CAT antagonist, Sigma-Aldrich), propargylglycine (PAG, 10 mM, CSE inhibitor, Sigma-Aldrich) and glybenclamide, (Gly, 10 μM, K_{ATP} channel blocker, Sigma-Aldrich) were added in the organ bath. After 15 minutes and 30 minutes for Gly, aorta rings were contracted with PE (1 μM) and a DHEA cumulative CCRC was performed. To confirm the involvement of NO, we used N^5-(1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO, 10 μM, eNOS inhibitor, Tocris). Finally, to assess if the relaxion was related to a direct action of DHEA or to his metabolization into T or E_2, we incubated aorta rings with Nil (10 μM, androgen receptor inhibitor, Sigma-Aldrich) and ICI 182,780 (100nM, high affinity oestrogen receptor, Tocris), antagonist, respectively for 15 and 30 minutes. Data were reported as % of relaxation respect to PE tone. Statistical evaluation was performed by using two-way ANOVA followed by Bonferroni as post hoc test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

3.5.5 In vitro cell culture experiments

BAEC were grown in Modified Dulbecco's Medium (DMEM) medium supplemented with 2 mmol/L glutamine, 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin/streptomycin, and incubated at 37°C in a 5% CO_2/95% air environment. The medium was changed every day until the cells become confluent. Passage 6-8 were used in all experiments. After serum deprivation for 24 h, the cells were treated with DHEA (1-10-100 nM) for 5 minutes with DMEM w/o phenol red.

3.5.6 NOx determination

Total nitrate content (NOx) that encloses NO_2^- and NO_3^-, was evaluated on medium of BAEC cells. The medium was incubated with STB (3 parts of NH4Cl 0,49M and 1 part of $Na_2B_4O_7$ 0,06M) in a microplate with cadmium (Sigma-Aldrich; 50 mg/well) for 1 hr in order to convert the inorganic anion NO_3^- to NO_2^- and then centrifuged at 12000 rpm at 4°C for 15 minutes [39]. We add the 2,3 diaminonaphtalene (DAN) which reacts with NO_2^- and gives the formation of the fluorescent product, 1-(H)-naphthotriazole. The reaction was terminated with the addition of NaOH 2.8 N. NOx was fluorometrically determined in microtiter plates by Promega Glomax explorer (Madison, WI) and calculated against a standard curve of sodium nitrite ($NaNO_2$, 50–2000 nM; Sigma-Aldrich). Data were reported as mean + SEM in μM concentration. Statistical evaluation was performed by using one-way ANOVA followed by Bonferroni as post hoc test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

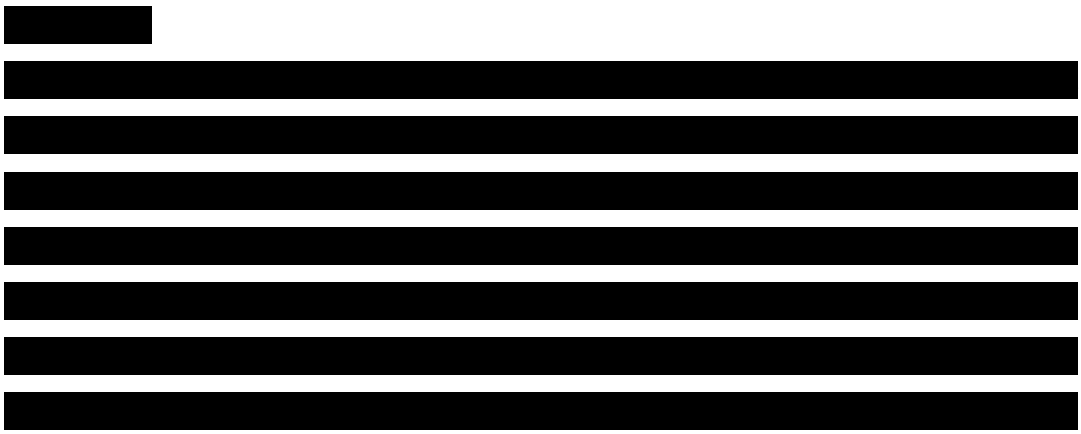
3.5.7 H_2S determination

H_2S production was assessed in the medium of cells as previously described [71]. Trichloroacetic acid solution (10% wt/vol, Carlo Erba Reagents) was added to each sample followed by zinc acetate (1% wt/vol Sigma-Aldrich). Next, N,N-dimethyl-p-phenylendiamine sulfate (20 mM, Sigma-Aldrich) and $FeCl_3$ (30 mM, Sigma-Aldrich) were added, and optical absorbance of the solutions was measured at a wavelength of 668 nm. All samples were assayed in duplicate, and H_2S concentration was calculated against a calibration curve of NaHS (3.9–250 μM). Data are reported as mean of μM concentration. Statistical evaluation was performed by using one-way ANOVA followed by Bonferroni as post hoc test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

3.5.8 H_2S determination in alive cells and aorta

In another set of experiment, we plated 10 000 BAEC cell/ well in 96-well black flat bottom and kept them growing for one day. The day after we changed the medium with DMEM w/o red phenol and after 30 minutes we add SF7AM (10 μM) at 37°C for 30 minutes in order to allow to quench H_2S. Thereafter we added DHEA (1 μM-100 nM) and we read fluorescence signal (Ex 475 nm, Em 500–550) at different scheduled times up to 60 minutes. The results were expressed as mean ± SEM. Statistical evaluation was performed by using two-way ANOVA followed by Bonferroni as post hoc test (GraphPad Software Inc., San Diego, CA).

For the aorta incubated with DHEA 100 μM the protocol was as follows: after the incubation aortic tissues were homogenized in modified RIPA buffer, protease inhibitor cocktail (Sigma-Aldrich) and sodium orthovanadate (10 mM). Protein concentration was determined by Bradford assay using bovine serum albumin as standard (Bio-Rad Laboratories, Milan, Italy). 50 μL of the sample at the concentration of 0.5 mg/mL was add to 145 μL of RIPA buffer and to 5 μL of SF7AM with a final concentration of 10 μM. After 1h we read fluorescence signal (Ex 475 nm, Em 500–550). All samples were assayed in duplicate, and H_2S concentration was calculated against a calibration curve of Na_2S (50nM – 200μM). The results were expressed as mean ± SEM. Statistical evaluation was performed by using t test (GraphPad Software Inc., San Diego, CA).



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in the absence of endothelium the relaxing effect of DHEA, in the presence of PAG or acid aspartic, was not modified.

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CHAPTER IV

Opa1 PROTEIN AND ENDOTHELIAL CELLS

4.1 Mitochondria

Mitochondria are the powerhouse of the cells where they produce ATP through oxidative phosphorylation (OXPHOS). Thus, it is the main organelle where oxidative reaction as Krebs cycles, beta-oxidation of protein, of fatty acid, and the OXPHOS is observed. The production of ATP is due to the passage of electrons from reduced coenzyme with high redox potential (NADH or $FADH_2$) to low (O_2) redox potential through the electron transfer chain (ECT) which is made of the complexes I to IV; complex I (NADH-ubiquinone oxidoreductase), II (succinate-dehydrogenease), III (ubiquinol-cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase). The main function of the ECT is to oxidize NADH and $FADH_2$ to NAD^+ and FAD^+ which will be used in the tricarboxylic acid cycle (TCA) to generate ATP, as shown in figure 30. This transport of electrons is followed by the pumping of H^+ from the mitochondrial matrix to intermembrane space that will use as a motive force to produce ATP. If during the electron transport there is a leak of electrons this can lead to a generation of ROS. The mitochondria have also many other functions as:

- Ca^{2+} buffering with the endoplasmic reticulum
- control of cell death through apoptosis
- ROS production
- Cell thermoregulation

They have a particular structure, with two membranes and these two membranes can delimit 3 spaces, the matrices, the intra-membrane space, and the cytoplasm. The internal membrane plea and form the crests. They have also the unicity to have their own genome and so a specific DNA called mitochondrial DNA (mtDNA). The formation of new mitochondria is regulated by the peroxisome proliferation-activated receptor gamma co-activator 1α (PGC-1α) which is a nucleus genome-encoded protein and a transcriptional activator of the nuclear respiratory factor

(Nrf1) [100]. Nrf1 is a transcription factor that has a role in the regulation of the transcription of some genes important for the replication and transcription of mtDNA, for example the mitochondrial transcription factor (TFAM), and proteins important in the respiratory chain. TFAM is the final effector of the activation for the duplication of mtDNA. PGC-1α has also an important role in antioxidant defense and regulates the cellular response to oxidative stress. The activation of PGC-1α is made from external stimuli for example Ca^{2+} or ROS. [101]. It has been shown that PGC-1α can have a negative control of vascular senescence [102]. Silent information regulator 2 homolog 1 (SIRT1) is a NAD-dependent deacetylase, it activates PGC-1α thanks to the lysine deacetylation. The deacetylation of PGC-1α results in the increase of its transcriptional function [103], as showed in figure 31.

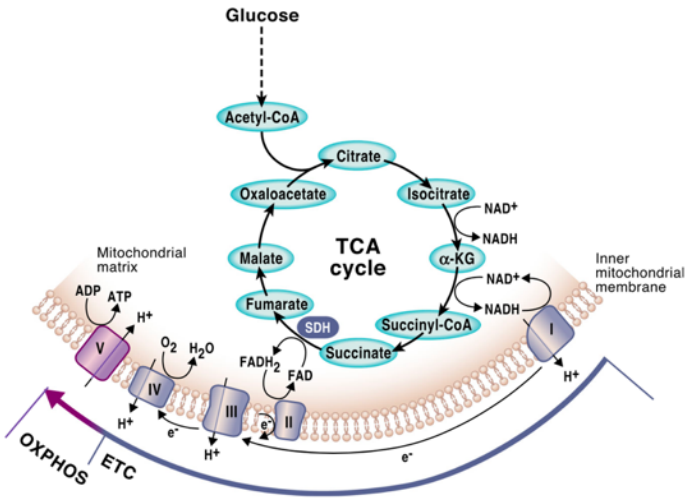

Figure 30: Mitochondrial TCA and ETC; the electron transfer chain (ECT) and oxidative phosphorylation (OXPHOS) [104].

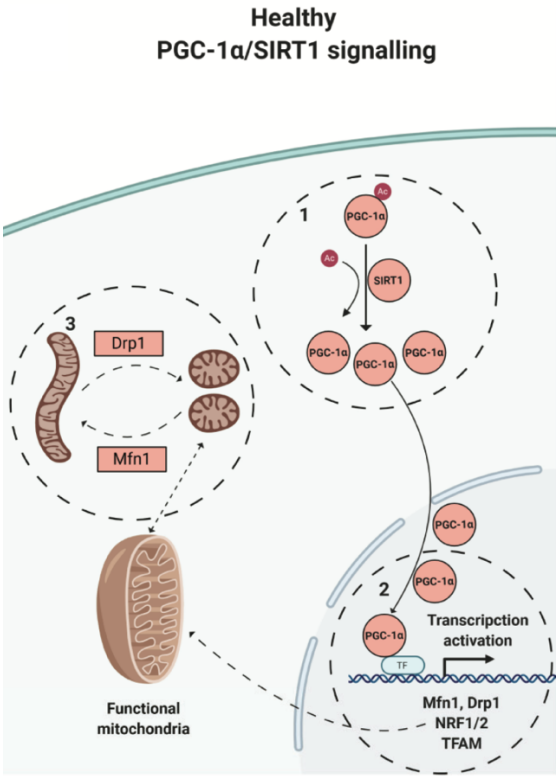

Figure 31: Healthy PGC-1α/SIRT1 signaling and mitochondrial dynamics, mitofusin-1 (Mfn1) indicating fusion, Dynamin-related protein 1 (Drp1), indicating fission. Modified image [105].

4.1.1 Mitochondria and endothelial cells

The mitochondrial density inside the cells is positively correlated to the need for energy; for example, skeletal and cardiac muscle cells, hepatic cells, and neurons contain a high number of mitochondria. By contrast, the EC has a specialty that has a smaller number of mitochondria. For example, in rat EC the mitochondria occupy only 2-6% of the volume of the cells compared to 32% in cardiomyocytes [106]. Indeed, the main source of energy for the EC is the glycolysis [107]. For example, 75% of ATP produced by pig aortic EC comes from glycolysis [108]. This is comparable to cancer cells and neutrophils [109]. The reason for this difference is due since ECs are exposed to the highest oxygen levels, and they facilized in this way the oxygen diffusion to perivascular cells using the minimum amount of oxygen and reducing the possible amount of ROS formation by using glycolysis [110]. So, mitochondria in EC don't have a primary role in ATP production but they have a role as sensors of the local environment [111]. They participate in cellular homeostasis and function and they also have a role in the production of ROS and in maintaining the Ca^{2+} concentration in the cytosol [112]. Moreover, NO is a reversible and competitive inhibitor of the complex IV of the phosphorylation chain, and so modulates the amount of oxidative metabolism [113], it inhibits the mitochondria membrane potential [114], the oxygen consumption and ATP production. In this context, importantly, mitochondria have a key role in the regulation of Ca^{2+} homeostasis and thus in NO production, meaning possible feedback from the mitochondria. This inhibition of cell respiration by NO and the activation of glycolysis involves the activation of the 6-phosphofructo-2-kinase [115].

4.1.2 Mitochondria dynamic

Mitochondria are not static organelles; they undergo continuous cycles of fusion and fission called mitochondrial dynamics, changing their form from elongated (fusion) to small and spheric (fission). Mitochondrial fusion is mediated by cytosolic guanosine-5'-triphosphate GTPases proteins mitofusin-1 (Mfn1) and mitofusin-2

(Mfn2) and optic atrophy 1 (Opa1) whereas fission is mediated by dynamin-related protein 1 (Drp1), a large cytosolic GTPase that is recruited by receptor protein fission 1 (Fis1) from the cytosol to mitochondria at the outer membrane [116]. Mfn1 and Mfn2 are present in the outer membrane while Opa1 is present in the intermembrane space (figure 32). The first step during fusion is the interaction of the Mfn1 and Mfn2 proteins followed by the interaction with the Opa1 protein. A balance between fission and fusion events is required for normal mitochondrial and cellular function. So, mitochondrial dynamics is important for the correct function and development of these organelles and also for programmed cell death [117]. A proper mitochondrial dynamic is important for the following mitochondrial function [118]:

- ⇒ ATP production
- ⇒ Cell development
- ⇒ Apoptosis
- ⇒ ROS generation
- ⇒ Functional of mtDNA

A change in this fragile balance can lead to a dramatic change in mitochondrial morphology and apoptotic cell death [119]. A reduction in fusion or an increase in fission leads to a fragmented phenotype whereas a reduction in fission or an augmentation in fusion leads to elongated tubules which are more efficient. It is important to remember that dysfunction in mitochondrial biogenesis and dynamic has an important impact on the energy supply and contributes to EC dysfunction and consequently to cardiovascular diseases.

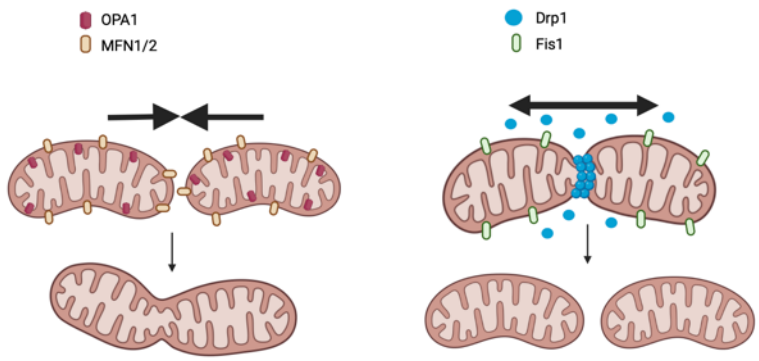

Figure 32: Mitochondrial dynamic; mitofusin-1 (Mfn1), mitofusin-2 (Mfn2), Optic atrophy 1 (Opa1), Dynamin-related protein 1 (Drp1) and fission 1 (Fis1). Created with BioRender.com.

4.1.3 Mitochondrial dynamic and EC in pathology

Whether fusion or fission is good or bad for ECs is not yet elucidated. For the moment, based on the scientific evidence, we can say that in many pathological conditions there is an augmentation of fission whereas fusion seams more protective. Indeed, fusion can be seen as positive because it facilitates metabolites protein and DNA diffusion in the mitochondrial network whereas the augmentation of fission is negative because it causes the fragmentation of mitochondrial with an increase of ROS [120]. In isolated mice arteries, Mdivi-1, a Drp1 inhibitor, thus an inhibitor of fission, increases ACh-dependent relaxation in an animal model of arterial hypertension (induced by chronic AngII perfusion), thus mitochondrial fission is involved in hypertension [121]. In diabetes, numerous studies shows that mitochondrial fission is increased. In type 1 diabetes, it has been shown in mouse coronary EC a reduction in Opa1 protein expression with an augmentation in Drp1 level. In line with these observations, diabetic mice present a rise in oxidative stress from both mitochondria and cytosolic origin. This can be corrected using an antioxidant, with a significant reduction of mitochondrial fragmentation [122]. Moreover, *Wang et al.* demonstrated that metformin, an antidiabetic medication, reduces Drp1 in type 1 diabetic EC, through AMPK and also improves endothelium-dependent relaxation and reduce atherosclerotic lesions [120]. Also, in rat retinal

ECs treated with high glucose, as a model of diabetic retinopathy, Drp1 and Fis1 protein increase in association with mitochondrial fragmentation. With the silencing of these two proteins, there is a reduction in oxidative stress, a reduction in mitochondrial fragmentation and an improvement in mitochondrial respiration [123]. Similarly, it was also demonstrated that in venous EC from a patient with type 2 diabetes there was an increased fission with smaller mitochondria, and ECs treated with high glucose shown more fission with an augmentation of mitochondrial oxidative stress and an impermeant of NO signaling, thus demonstrating that mitochondrial fission is involved in endothelial dysfunction in diabetes [124]. Also, in EC isolated of human arterioles from type 2 diabetic patients it has been shown a reduction in mitochondrial mass suggesting an increased fission and an increase mitochondrial ROS production [125]. Altered mitochondria dynamics has also been evidenced in cardiac diseases. After ischemia/reperfusion, there is an increase in fission (mediated by Drp1), as well as in NO and ROS production in human EC [126]. In a model of atherosclerosis, treating rat ECs with palmitate, induces an increase in mitochondrial fission with Drp1 overexpression leading to excessive ROS production [127]. Moreover, in this same condition has been seen that Drp1 and NADPH oxidase 2 (NOX_2) regulate each other and that the inhibition of Drp1 can be a strategy to suppress oxidative stress [128]. It has also been found that in mouse Light Density Lipoprotein receptor KO (LDLr $^{-/-}$ mice prone to atherosclerosis when fed a high-fat diet), that lack of Opa1 in EC increases the lipid deposits, showing a protective role for endothelial Opa1 against atherosclerosis [129]. In conclusion, we can say that in pathological condition mitochondrial fission increases, as shown in figure 33 and in table 1 but more studies are needed to clarify the protective role of fusion in ECs.

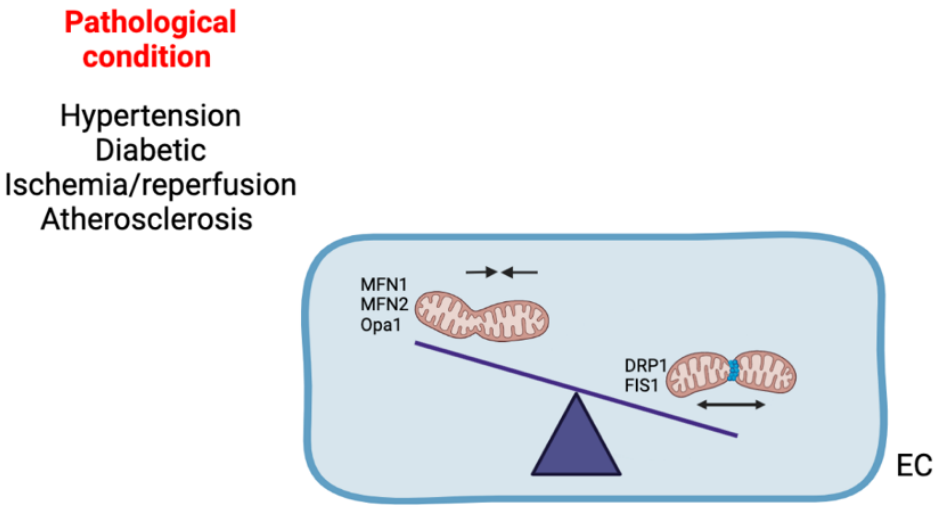

Figure 33: Mitochondrial dynamics in pathology condition in EC; mitofusin-1 (Mfn1), mitofusin-2 (Mfn2), Optic atrophy 1 (Opa1), Dynamin-related protein 1 (Drp1) and fission 1 (Fis1). Created with BioRender.com.

Pathology	Model	Effect in fusion and fission
Diabetes Type 1	MCEC from diabetic mice [122]	↑ $O_2^{\bullet-}$ → ↓Opa1 ↑Drp1
	HUVEC+HG/ Diabetic mice [120]	↑ Drp1
	RREC + HG [123]	↑ Drp1/Fis 1 → Mitochondrial fragmentation
Diabetes Type 2	EC from patientes/HAEC [124]	↑ Drp1/Fis 1 ↑ mROS
	EC from human arterioles [125]	↓ mitochondrial mas ↑ mROS
Hypertension	C57 mice + AngII [121]	↑ Fission ↓ NO
Ischemia/reperfusion	HUVEC + I/RP [126]	↑ Drp1
Atherosclerosis	RAEC + palmitate [127;128]	↑ Drp1 Drp1 ↔ NOX_2
	$LDLr^{-/-}$EC-$Opa1^{-/-}$ [129]	EC-$Opa1^{-/-}$ → ↑ Atherosclerosis

Table 1: EC model and correlation with mitochondrial dynamic in different pathologies; mouse coronary endothelial cell (MCEC), human umbilical endothelial cell (HUVEC), high glucose (HG), rat retinal endothelial cell (RREC), human aortic endothelial cell (HAEC), ischemia/reperfusion (I/RP), rat aortic endothelial cell (RAEC). Created with BioRender.com.

4.2 Endothelial cells aging and mitochondrial function

Aging is the most important risk factor for CVDs [130]. In the aged human EC, it has been shown a reduction in both mitochondrial fusion and fission mechanisms [131]. In contrast with this result, it has been demonstrated that in senescent ECs there is a reduction in Drp1 that stimulates the production of ROS [132]. Moreover, in the same senescent cells, a ROS stimulus could lead to a protective response

through a decrease of Drp1 and Fis1, protecting the mitochondria from the fragmentation and loss of mitochondrial membrane potential [133]. In this context, the free-radical theory of aging is based on the fact that ROS damage the biological macromolecules leading to a functional decline [134]. Moreover, ROS formation can damage the mtDNA and the respiratory chain and increase the ROS production by the mitochondria. Thus, mitochondrial biogenesis diminishes with age, leading to a mitochondrial dysfunction in aging [135]. So, more studies are needed to clarify the role of mitochondrial dynamics in the aged ECs keeping in mind the free-radical theory.

4.3 Opa1 protein and its role in the vascular system

Opa1was first identified in a neurodegenerative disease known as the autosomal dominant optic atrophy disease characterized by a mutation of Opa1 protein [136] that induces progressive and irreversible vision loss. Opa1 has a major role in the organization of mitochondrial internal membrane and in fusion process. This protein has also an important role in the energetic metabolism and in the organization of the mitochondrial crest. In fact, a loss of Opa1 leads to a disorganization of cristae and mitochondrial fragmentation with a mitochondrial dysfunction as a reduction of ATP production, an alteration in Ca^{2+} homeostasis and an alteration in the regulation of cell growth and apoptosis [137]. This mitochondrial alteration could lead to an excessive in ROS production [135]. Moreover, Opa1 has also other roles as the controlling of the mitochondrial respiratory activity by the stabilization of respiratory chain super-complexes [137]. A defect in mitochondrial network has been observed in most cardiovascular disorders and in ECs aging. A reduction in Opa1 (Opa1 haploinsufficiency model $Opa1^{+/-}$) level is associated with a higher arterial hypertension due to L-NAME treatment with a reduction in endothelial-dependent relaxation and an increase of ROS formation, underling a new protective role for Opa1 in the vasculature [138]. Recently, it has been observed that the absence of Opa1 in human ECs reduced the vascular response to flow (shear stress) by

increasing of ROS [129]. Finally, more information is needed to understand the protective role of Opa1 role in EC also related to the changes associated with aging.

4.4 Oxidative stress in endothelial cells

ROS included $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), radical hydroxyl ($^{\bullet}OH$), peroxynitrite ($ONOO^-$) and hydrochloride acid (HOCl). All the vascular cells can produce ROS and they are toxic for the cell when they are produced in excess. In normal conditions they are important for cell proliferation and differentiation while an excess in ROS production became toxic.

They have several sources of ROS:

- NOXs
- mitochondrial electron transport chain ETC
- xanthine oxidase
- uncoupled eNOS
- cytochrome P450

In EC, ROS come from NOX_2 and NOX_4 isoform and from the mitochondria. NOX_2 is composed of six subunits, gp91, $p22^{phox}$, Rac1, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ whereas NOX_4 is formed by p22, POLDIP2 and calnexin. In NOX_2 gp91 and $p22^{phox}$ are membrane proteins while $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac1 are cytosolic proteins. In response to a cellular stress the cytosolic part moves to the membrane and activates NOX oxidase. ROS are produced also by complex I and III of the mitochondria, thus during oxidative phosphorylation which involves the transfer of an electron to a molecule of oxygen. In the physiological condition the molecule of O_2 is converted into H_2O and only 5% is converted in $O_2^{\bullet-}$ [139]. Several mechanisms acting to protect cells in a condition of oxidative stress are available. Among them, superoxide dismutase (SOD) is the main enzymes that protect cells from oxidative stress. There are two SODs called Cu/ZnSOD, which is cytosolic, and the mitochondrial dismutase called MnSOD. The SODs transform $O_2^{\bullet-}$ in H_2O_2 that is

converted in H_2O by catalase in the cytosol and by glutathione peroxidase (in the mitochondria and in the cytosol) [140]. In various cardiovascular diseases, the antioxidant defense cannot heal the ROS production which becomes excessive for the cell, and this process is called oxidative stress [141]. Oxidative stress is an important component of some of the most harmful cardiovascular disease as arterial hypertension, cardiac hypertrophy and atherosclerosis. In ECs, ROS promotes endothelial senescence [142] and cellular membrane hyperpermeability [143] leading to the impairment of the endothelial barrier function. Moreover, vascular ROS induce vascular inflammation and endothelial dysfunction. As already said, NO interacts with $O_2^{-\bullet}$ and form $ONOO^-$ that can cause nitration of protein in an irreversible way. Thus, ROS produced by the mitochondria (mtROS) could contribute to cardiovascular diseases as suggested by the observation that Mito-TEMPO, an inhibitor of the mitochondrial production of $O_2^{\bullet-}$, inhibits Ang II induced arterial hypertension, increasing the vascular NO and thus improving NO relaxation [144].

4.5 Renal microcirculation

Anatomically, the kidney is divided into capsule, cortex region and medullary region. The renal artery enters the kidney and separates into segmental, interlobar, arcuate, and interlobular arteries and became the glomerular capillaries where fluid and solutes are filtered (figure 34). The kidney has a unique and complex vascular circulation, it is a double capillary bed, once in the glomerulus and once in the tubules which have an important role in filtration. Thus, the kidney is characterized mostly by microcirculation. The microcirculation is the terminal systemic circulation consisting of microvessels with diameters <20 μm. These microvessels consist of arterioles, glomerular capillaries, peritubular capillaries post-capillary and venules. Microcirculation is having a vital role in the function of the organ for the transportation of oxygen and nutrients and the removal of toxins. The kidney microvasculature is complex because of the extreme environmental variations with

changes in oxygenation and osmolality from the cortex to the outer medullary region. Vessels of the microcirculation are almost entirely lined by EC [145]. Each organ benefits from a unique endothelium with a specific function [145]. The kidney has one of the richest and most diversified EC population. This extensive diversity can be explained on two counts, firstly, renal endothelium contributes to differential transport capabilities across the various segments of the nephron and secondly, the endothelium must adapt to different in oxygenation and osmolality from cortex to medullary region [148]. Experimental evidence has demonstrated that renal microvascular dysfunction is associated with CVD but is not yet clear if it is a consequence or a cause [145]. Indeed, the kidney is designed as the target organ often affected very early in cardiovascular diseases demonstrated by an enhanced cardiovascular risk associated with microalbuminuria which is the persistent elevation of albumin in the urine [146]. Loss of the microvasculature network has been linked to progressive loss of kidney function and acute kidney injury [149, 145]. The study of renal EC may aid to identify new target for the treatment of kidney disease and kidney regeneration.

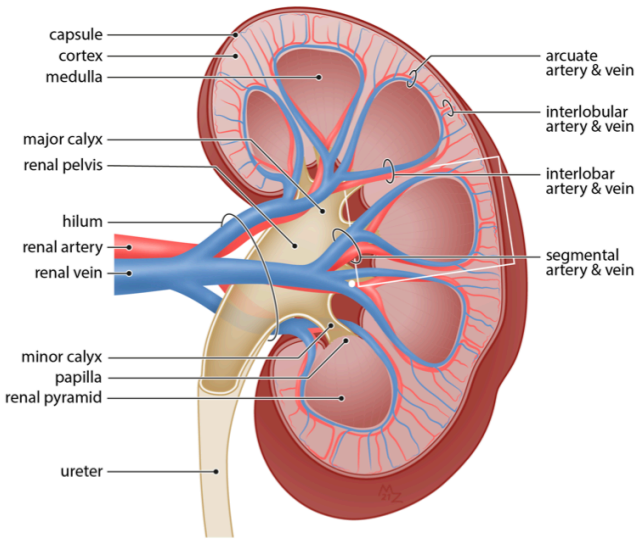

Figure 34. Anatomy of the renal vasculature [150]

4.6 Aim

Endothelial dysfunction is the hallmark of cardiovascular pathologies. In ECs, mitochondria are more involved in the regulation of cell homeostasis than in the production of ATP. As reported before, Opa1 has a protective role in the vascular system [129, 138]. So, we hypothesized that a reduction in Opa1 protein expression level specifically in the ECs could affect the endothelial functions and hence the vascular function. In addition, reducing Opa1 level in EC could participate into the endothelial dysfunction that occur in aging. Thus, this third part of the thesis aimed to investigate the role of Opa1 protein in the endothelium with an overview in aging ECs. To this aim we used KO mice lacking Opa1 exclusively in ECs. The expression levels of proteins important in the endothelial function, in the oxidative stress, in inflammation and in mitochondrial function were evaluated in the aorta (conducting vessels), in the mesenteric artery (resistance artery) and in the kidney (microcirculation). We also analyzed the functionality of the aorta and of the mesenteric artery.

4.7 Material and methods

4.7.1 Animals

We used a mouse model of Opa1 KO in EC (EC-Opa1) were generated by crossing Cadherin5-CreERT2^{+} mice with Opa$^{loxP/loxP}$ mice. Whereas the EC-WT mice were generated by crossing Cadherin5-CreERT2^{-} mice with Opa$^{loxP/loxP}$ mice, as we can see in the figure 35. The mice were all housed in a temperature-controlled room with a 12h/12h day/night cycle and had access to water and standard animal food *ad libidum*. All procedures were performed in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM) and were approved by the local Animal Care and Use Committee (APAFIS#3577-201601141342229, APAFIS#2018011217209, APAFIS#30385-202103101014575O). The investigation conforms to the directive 2010/63/EU of the European Parliament. The deletion was induced by injection of tamoxifen

(150mg/kg per day, for 5 consecutive day) and mice were used after 5 weeks. These KO mice were generated by breeding the Cre-driven strain with the floxed mouse. This technique is called inducible Cre-loxP system and it is a technology widely used for gene editing. Cre recombinase recognizes a specific DNA fragment sequence called loxP and then mediates the site-specific deletion of DNA sequence between two loxP. Cre needs a promoter that target a specific cell which is cadherin in our study. Tamoxifen, which binds to estrogen receptor bound to Cre, allow Cre to move inside the nucleus and recognize the loxP sequence, as in figure 36. The Opa1 silencing was 61% in EC from resistance mesenteric arteries isolated from EC-Opa1 mice [129]. We used adult (8 months) and old (22 months) mice lacking Opa1 in ECs (EC-Opa1$^{-/-}$) and their littermate controls (EC-WT). Kidneys, major target organ in cardiovascular diseases, aorta and mesenteric arteries were collected for western-blot analysis and for functional study.

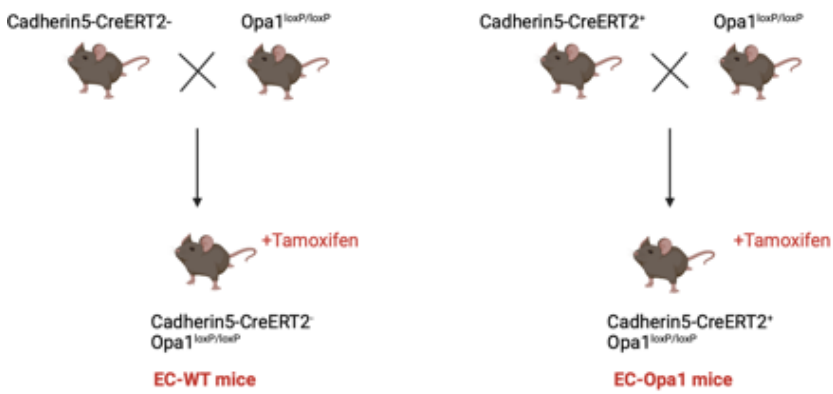

Figure 35: Crossin between Cadherin5-CreERT2^{+} mice with Opa1$^{loxP/loxP}$ mice and Cadherin5-CreERT2^{-} mice with Opa1$^{loxP/loxP}$ mice. Created with BioRender.com.

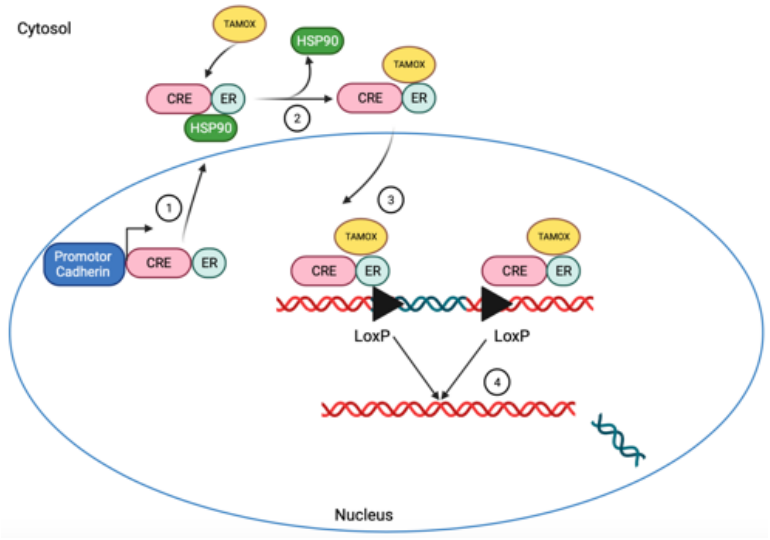

Figure 36: Inducible Cre-loxP system; Tamoxifen (TAMOX); Cre recombinase (Cre); estrogen receptor (ER). Created with BioRender.com.

4.7.2 Analysis of protein expression levels by Western blot

After euthanasia of the animal, the abdominal aorta, the mesenteric artery and the kidneys were isolated from adult mice and old mice EC-Opa1$^{-/-}$ and aged-matched littermate control EC-WT and place in liquid nitrogen (N_2).Kidney, aorta and mesenteric artery proteins were extracted in SDS lysis buffer with 20% SDS, Tris 500 mM pH7.4, 0.5 M EDTA solution (cat #1861283 Thermo Fisher scientific) and with protease and phosphatase inhibitor cocktail (cat #78444 Thermo Fisher scientific). Homogenates were centrifugated at 14000 rpm at 8°C for 20 minutes and the supernatant was collected. Protein concentration was determinated by the Micro BCA protein essay kit (cat#23227 Thermo fisher scientific). The equal amount of protein was used, 30 µg for kidney, 10 µg for mesenteric artery and aorta. This amount of protein was processed to denaturation by using Laemmli sample buffer containing 5% beta-mercaptoethanol, boiled 5 minutes at 95°C and after separated in 4-15% polyacrylamide gel electrophoresis (#5678085 Bio-Rad) and transferred in 0.2 µm nitrocellulose membrane (cat#1704159 Bio-Rad). The membrane was incubated for 1h in Tris-buffered saline with 0.1% Tween® 20 detergent (TBST) bovine serum albumin (BSA) 5 % for the blocking solution. After overnight at 4°C with the primary antibody (see Table 2) followed by the secondary antibody for 1 h at room temperature (Sigma-Aldrich A05445 Anti Rabbit IgG 1/3000). The reaction was visualized by ECL detection (cat#170-5061 Bio-Rad). Bands were quantified using a Chemi Doc (Bio-Rad) chemiluminescent detection system and densitometrical analyzed using Image Lab (Bio-Rad). All the information regarding antibodies, source and experimental conditions are summarized in table 2. The target protein band intensity was normalized over the intensity of the housekeeping protein GAPDH. Data are expressed as mean ± SEM. Statistical evaluation was performed by using Two-Way ANOVA followed by a Bonferroni's multiple comparisons test or two-tailed Mann-Witney test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

Target antigen	Source	Catalog #	Working concentration
eNOS	BD bioscience	BD610297	1/1000
iNOS	BD bioscience	BD 610432	1/2000
p67phox	ABclonal	A1178	1/1000
p47	BD bioscience	BD610355	1/500
VCAM	Cell signaling	#13662	1/1000
p22phox	Cell signaling	#27297	1/1000
TFAM	Invitrogen	18G102B2E11	1/1000
MnSOD	Cell signaling	#13141	1/1000
CD45	Abcam	Ab10558	1/500
gp91	BD bioscience	BD611414	1/1000
Cu/ZnSOD	Enzo	ADI-SOS-101-E	1/1000
GAPDH	Cell signaling	#5174	1/2000
ICAM-1	Santa Crouz	Sc-8439	1/200
PGC-1α	Abcam	Ab106814	1/1000
Nrf1	Cell signaling	#46743	1/1000
CSE	Cell signaling	# 19689	1/1000
3-nitrotyrosine	Abcam	Ab61392	1/1000
SIRT1	Cell signaling	#9475	1/500
peNOS (pSerine1177)	BD bioscience	BD612392	1/1000
3-MST	NOVUS	NBP1-54734	1/1000
Cav-1	BD bioscience	BD610407	1/1000

Table 2: Antibody types, source and experimental condition used for western blot analysis.

4.7.3 OXYBLOT

We use the protein oxidation detection kit (Oxyblot, Merk 87150). In this kind of immunoblot it is possible to detect the presence of carbonyl groups which are a markers of protein oxidation. To this aim, 20 µg of protein of kidney were used; in

one tube, we perform the derivation reaction by reacting the samples with 2,4-dinitrophenylhydrazine (DNPH) that will form the 2,4-dinitrophenylhydrazone (DNP-hydrazone) by the interaction with the carbonyl group of the samples. In another tube, used as negative control, were measured the reaction with the control solution. After 15 minutes of incubation, the neutralization solution was added to stop the reaction. Then we perform an SDS-PAGE with a 12% SDS polyacrylamide gel and we electroblotted onto nitrocellulose membrane. We incubate the membrane with blocking/dilution buffer for 1 hour. The detection of the carbonyl group was allowed by using the primary antibody Rabbit Anti-DNP incubated overnight, after washing, by the addition of the secondary antibody Goat anti-Rabbit IgG (HRP-conjugated). Data are expressed as mean ± SEM. Statistical evaluation was performed by using two-tailed Mann-Witney test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

4.7.4 Ex vivo vascular reactivity, myography

After euthanasian of the animal, the thoracic aorta and the mesenteric artery from adult mice and old mice EC-Opa1$^{-/-}$ and aged-matched littermate control EC-WT were collected and placed in physiological salt solution (PSS) (NaCl 130 mM, KCl 3.7 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 14.9 mM, $CaCl_2$ 1.6 mM, HEPEX 5 mM, KH_2PO_4 1.2 mM, D-glucose 11 mM) at pH 7.4 thanks to bubbling (pO_2 160 mmHg and pCO_2 37 mmHg) at 37°C. Segments of second order mesenteric resistance arteries (2 mm long) were mounted on a 610 M wire-myograph (Danish Myo Technology, Aarhus Denmark). Two tungsten wires (40 µm diameter) were inserted into the lumen of the arteries and fixed to a force transducer. For the mesenteric artery the tension was increased of 0,6 mN to obtain 90 mmHg (equivalent to the intra-arterial pressure), whereas for the aorta was increased of 1 mN to 90 mmHg. After 45 min equilibration, the artery was tested twice with potassium rich PSS (80 mmol/L). The vascular functionality testing of the aorta and in mesenteric artery followed the same protocol. On stable tone of PE (1 µmol/L), a single concentration

of ACh (1µmol/L) was added to evaluate the integrity of the endothelium in the different group. Thereafter a cumulative concentration curve of ACh (1 nmol/L to 100 µmol/L) was performed on a stable tone of PE (1 µmol/L) in the absence or in the presence of the following drugs for 20 minutes:

- Mito-TEMPO, inhibitor of the production of mitochondria $O_2^{\bullet-}$ (10 µM)
- Indometacin (INDO), which is a non-selective inhibitor of cyclooxygenase (COX-1 and COX-2) (10 µM)

A CCRC to PE (1 nmol/L to 100 µmol/L) was also performed to evaluate the smooth muscle contractility. Data are expressed as mean ± SEM. Statistical evaluation was performed by using Two-Way ANOVA followed by a Bonferroni's multiple comparisons test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

4.7.5 Perfused isolated mouse kidney and mensuration of NOx

In a different set of experiments the right renal artery was cannulated mice under anesthesia and the kidney was collected and perfused with PSS at 37°C as describe previously [129]. The perfusion pression was measured continuously (PM-4 pressure monitor LSI, Burlington VT, USA) and the PSS perfusing the kidney was collected when flow was at 600 µl/min. The perfusate was frozen in liquid N_2 and stored at 80°C. Thereafter NOx by Nitrate/nitrite fluorometric assay kit (Cayman 780051) was measured. Data are expressed as mean ± SEM. Statistical evaluation was performed by using two-tailed Mann-Witney test by using GraphPad Instant (GraphPad Software Inc., San Diego, CA).

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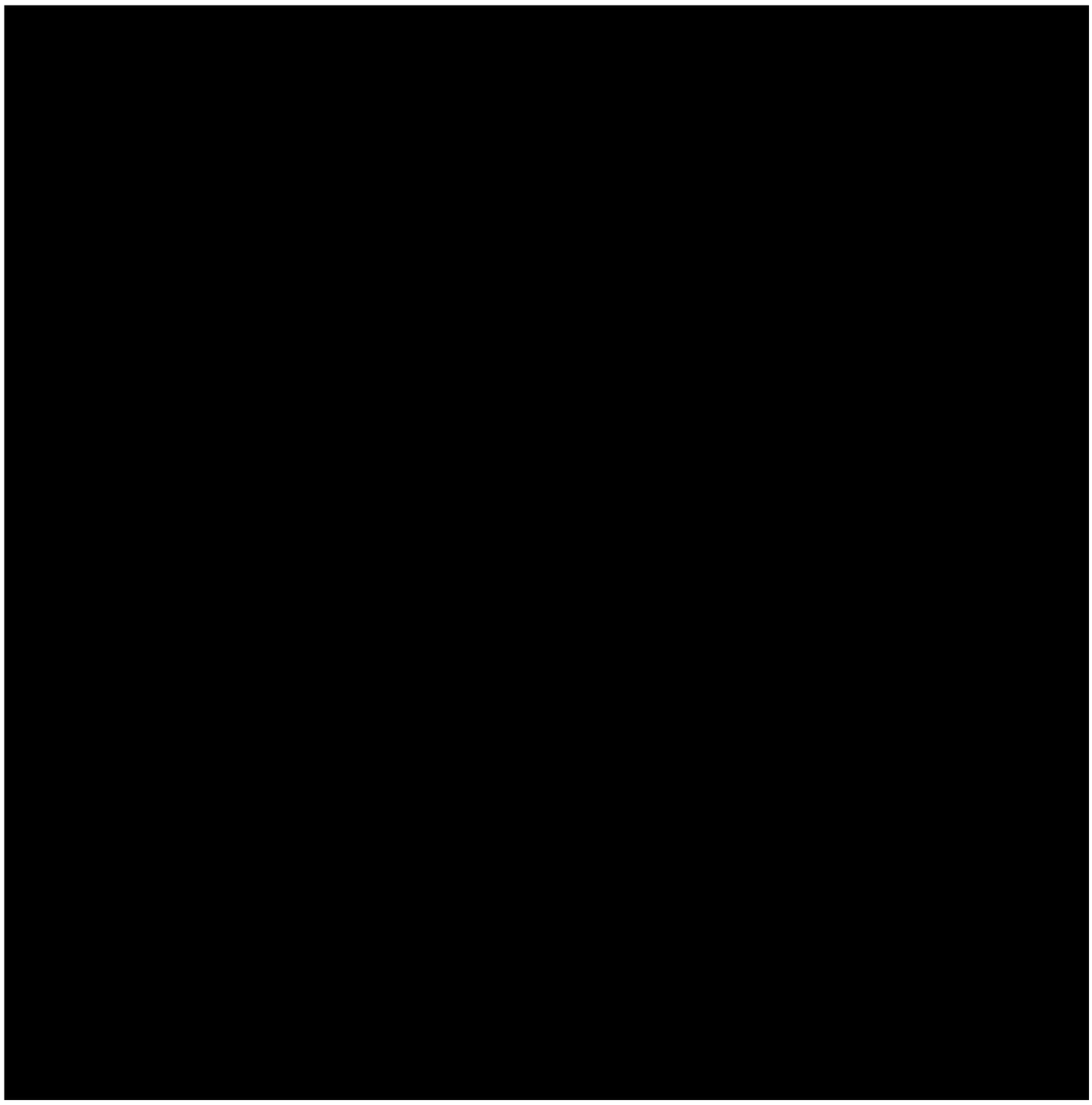

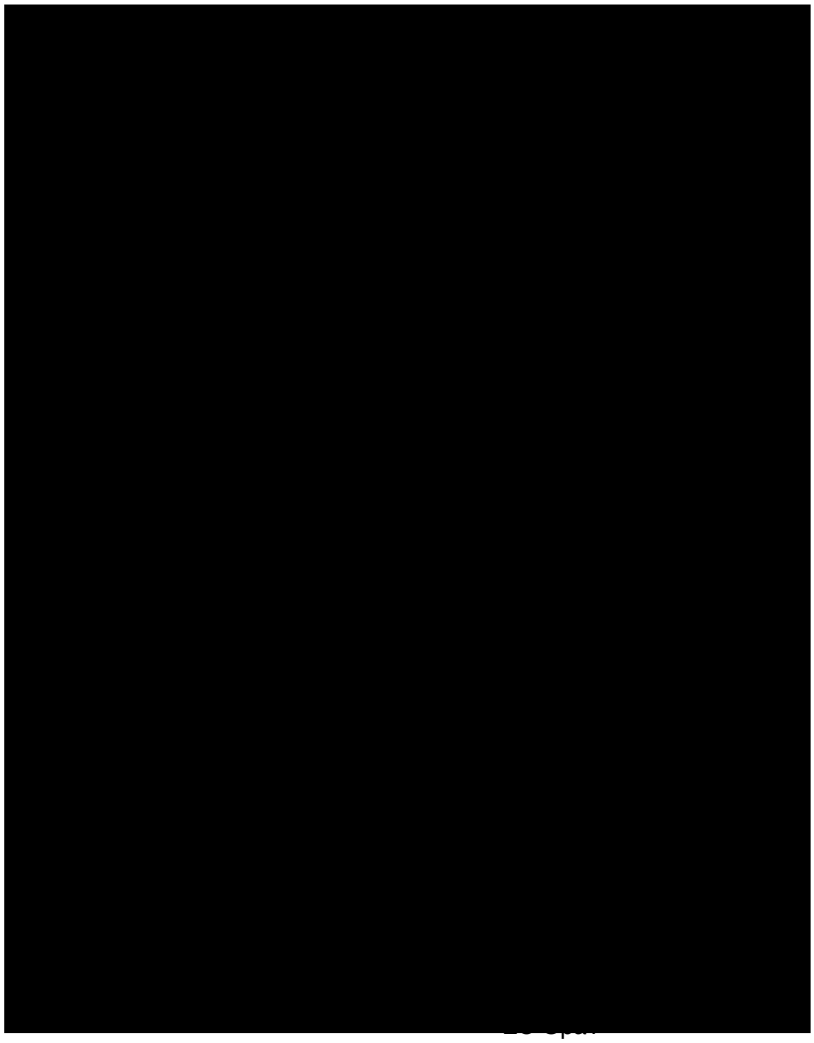

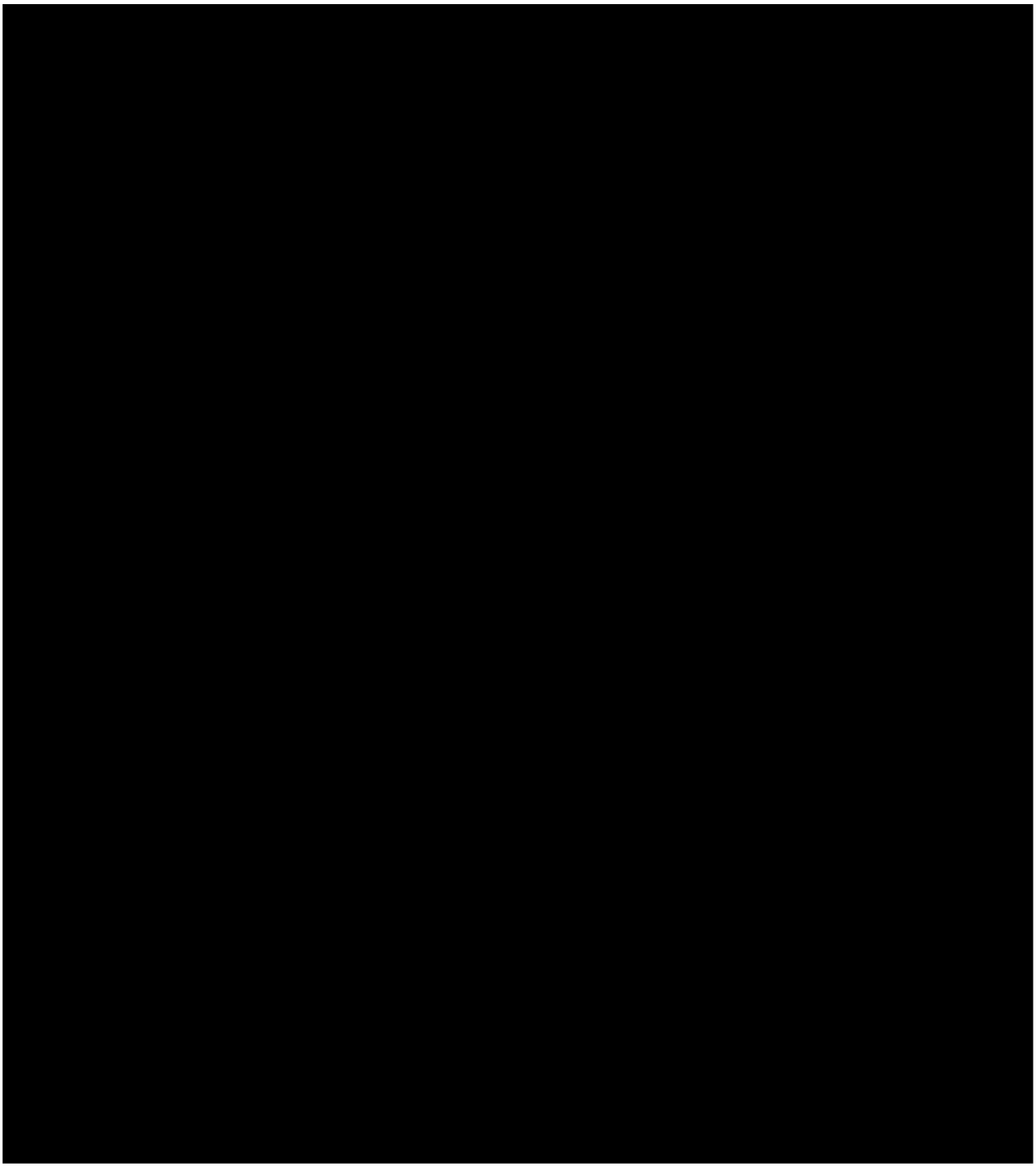

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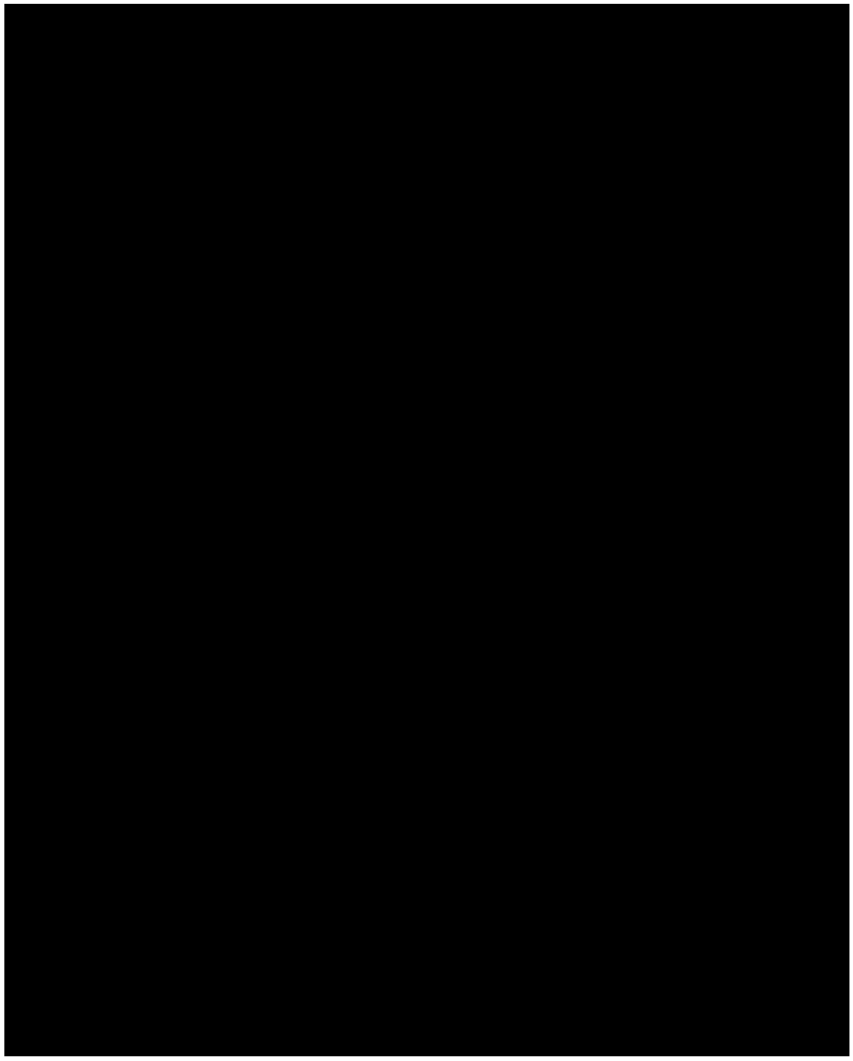

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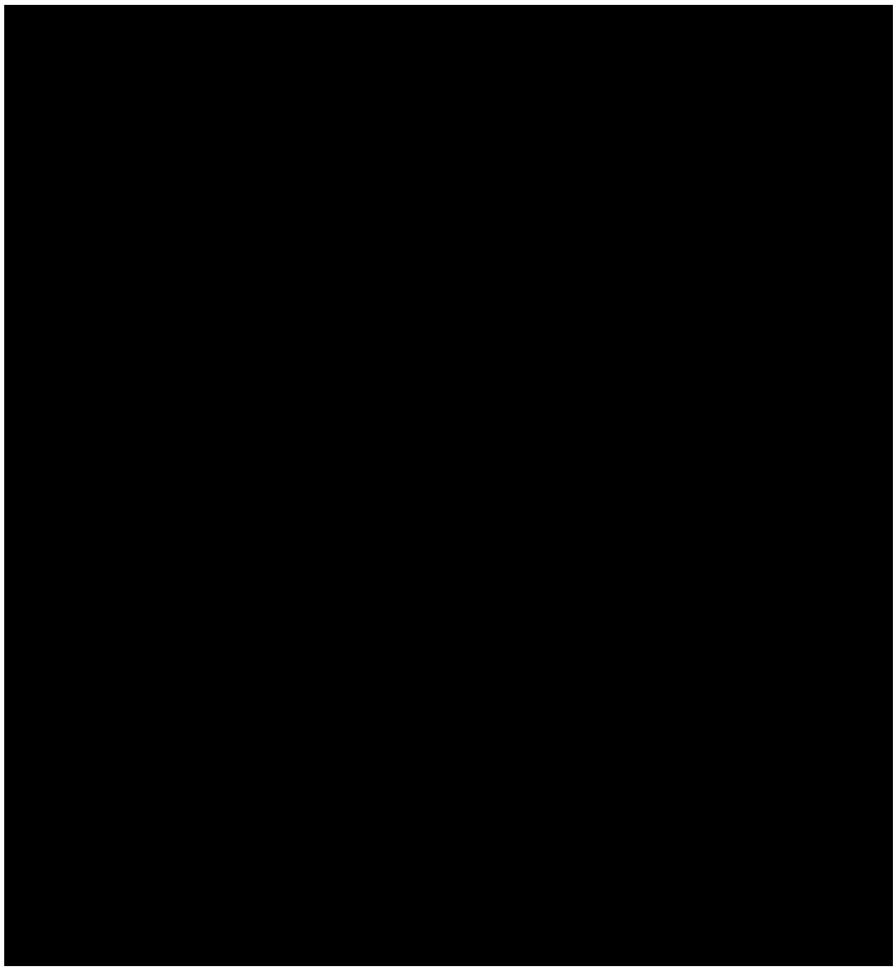

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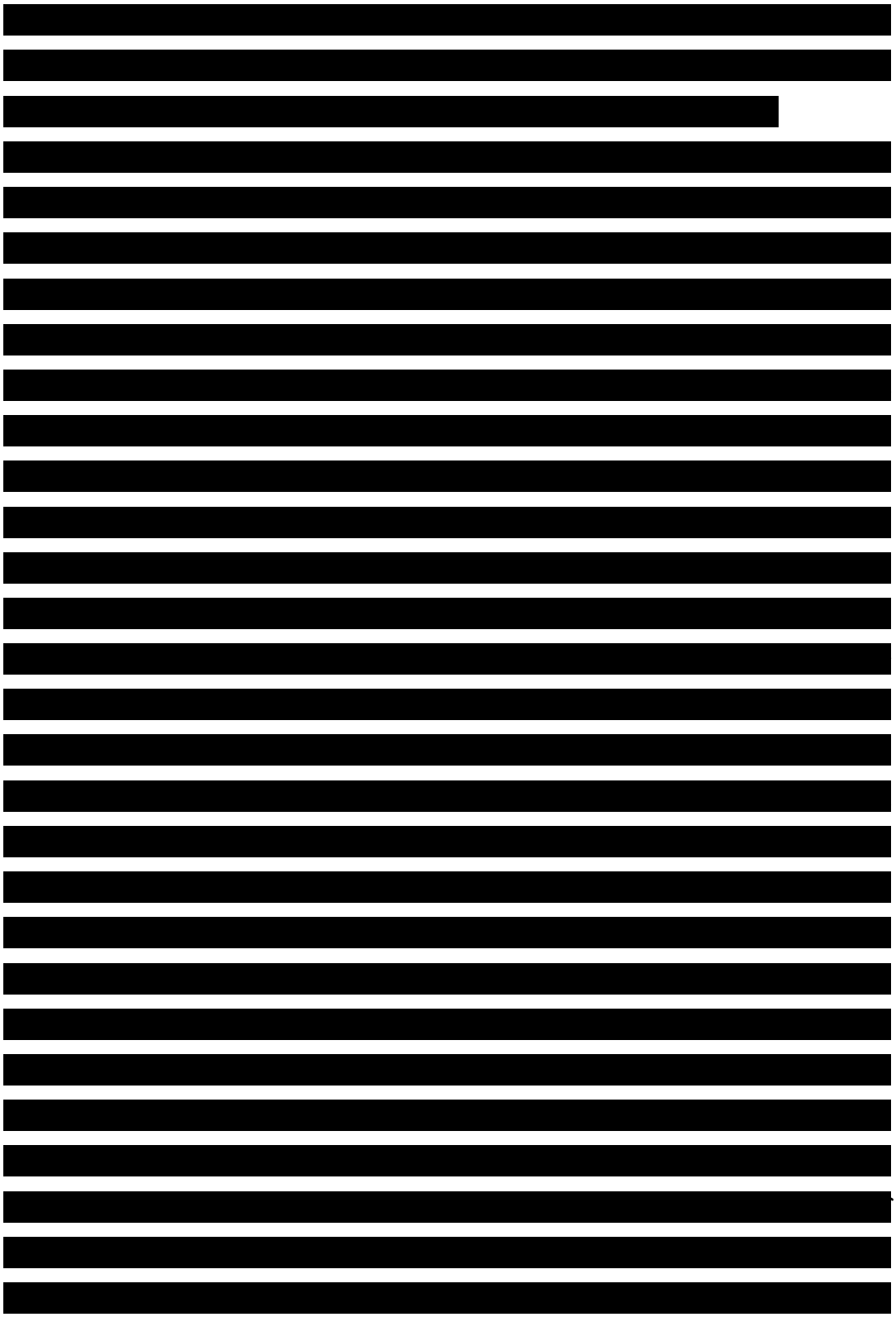

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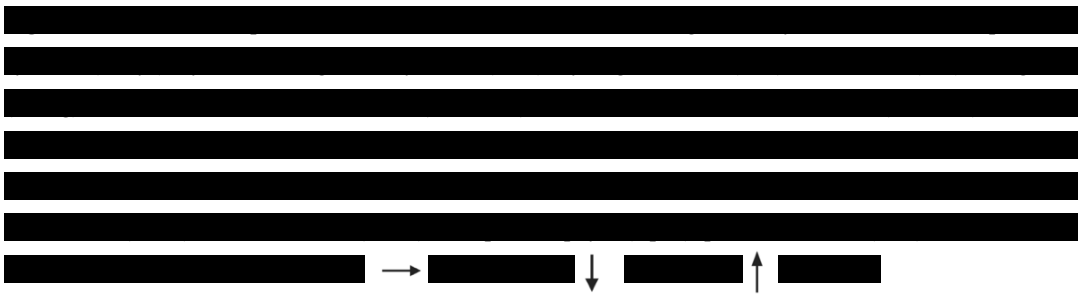

CONCLUSIONS AND FUTURE DIRECTIONS

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