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Altered mitochondrial dynamics induce endothelial dysfunction and increase atherosclerosis in mice

Ahmad Chehaitly

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L'UNIVERSITE D'ANGERS

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Par

« **Ahmad CHEHAITLY** »

« **ROLE OF MITOCHONDRIAL DYNAMICS IN THE RESPONSE OF
ENDOTHELIAL CELLS TO SHEAR STRESS DURING EARLY PHASE
OF ATHEROSCLEROSIS** »

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“Great things are not done by one person.

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Steve Jobs

*To the victims of Covid-19, victims of 04 August 2020 Beirut Port explosion, and
to my parents, I dedicate this work...*

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List of Abbreviations

Abbreviation	Long Form
AA	<i>Arachidonic Acid</i>
AAo	<i>Ascending Aorta</i>
AbAo	<i>Abdominal Aorta</i>
ACE	<i>Angiotensin Converting Enzyme</i>
Ach	<i>Acetyl-Choline</i>
ADP	<i>Adenosine Di-phosphate</i>
AGT	<i>Angiotensinogen</i>
AKT	<i>Protein Kinase B or PKB</i>
AMPK	<i>AMP-activated protein kinase</i>
Ang II	<i>Angiotensin II</i>
AoA	<i>Aortic Arch</i>
AP-1	<i>Activator Protein 1</i>
Apo1	<i>Apoptosis Antigen 1</i>
ATP	<i>Adenosine TriPhosphate</i>
ATP	<i>Adenosine Tri-Phosphate</i>
ATR	<i>Angiotensin Receptor</i>
BAK	<i>BCL-2 Antagonist Killer</i>
BAX	<i>BCL (B Cell Lymphoma)-Associated X</i>
BCL-2	<i>B-cell lymphoma 2</i>
BH4	<i>(6R) -5,6,7,8-tetrahydro-L-biopterin</i>
BK	<i>Bradykinin</i>
Ca ²⁺	<i>Calcium</i>
Caf4	<i>CCR4-associated factor 4</i>
CaM	<i>Calmodulin</i>
cAMP	<i>Cyclic Adenosine MonoPhosphate</i>
Cav-1	<i>Caveolin 1</i>
Cox	<i>Cyclooxygenases</i>
CU	<i>Calcium Uniporter</i>
CVDs	<i>CardioVascular Diseases</i>

Cyp1b1	<i>Cytochrome P450 1 B1</i>
DAG	<i>Diacylglycerol</i>
DAPI	<i>Diamidino-2-PhenylIndole</i>
DNA	<i>Deoxy-ribonucleic Acid</i>
Dnm2	<i>Mitochondrial fission GTPase Protein</i>
DRP1	<i>Dynamin Related protein 1</i>
EC	<i>Endothelial Cells</i>
ECE	<i>Endothelin-Converting Enzyme</i>
EDCF	<i>Endothelium Derived Contracting Factor</i>
EDHF	<i>Endothelium Derived Hyperpolarizing Factor</i>
Edn1	<i>Endothelin 1</i>
EDRF	<i>Endothelium Derived Relaxing Factor</i>
EEL	<i>External Elastic Lamina</i>
eNOS	<i>Endothelial Nitric Oxide Synthase</i>
ER	<i>Endoplasmic Reticulum</i>
ERK	<i>Extracellular signal-Regulated Kinases</i>
ET-1	<i>Endothelin I</i>
ET-AR	<i>Endothelin A receptor</i>
ET-B	<i>Endothelin Beta</i>
ET-BR	<i>Endothelin B receptor</i>
ETC	<i>Electron Transport Chain</i>
FADH	<i>Flavin Adenine Dinucleotide</i>
FAK	<i>Focal Adhesion Kinase</i>
Fis-1	<i>Fission 1</i>
FMD	<i>Flow Mediated Dilatation</i>
GC	<i>Greater Curvature</i>
GPCR	<i>G-Protein Coupled Receptor</i>
GTP	<i>Guanosine Tri-Phosphate</i>
GPXs	<i>Glutathione Peroxidases</i>
HAEC	<i>Human Aortic Endothelial Cells</i>
HF	<i>High Flow</i>
HFD	<i>High Fat Diet</i>
HUVEC	<i>Human Umbilical Vein Endothelial Cells</i>
ICAM-1	<i>Intracellular Adhesion Molecule 1</i>

IEL	<i>Internal Elastic Lamina</i>
IL-1	<i>Interleukin 1</i>
IMM	<i>Inner Mitochondrial Membrane</i>
IP-3	<i>Inositol 1,4,5-trisphosphate</i>
IP3R	<i>Inositol 1,4,5-trisphosphate</i>
JAM-1	<i>Junction Adhesion Molecule 1</i>
JNK-1	<i>Stress-activated protein kinase jnk-1</i>
KCl	<i>Potassium Chloride</i>
KDa	<i>Kilo Dalton</i>
Klf-2	<i>Kruppel Like Factor 2</i>
KO	<i>Knock Out</i>
LC	<i>Lower Curvature</i>
LDL-r	<i>Low Density Lipoprotein Receptor</i>
LF	<i>Low Flow</i>
L-NAME	<i>L-Nitro-Arginine Methyl Ester</i>
LSS	<i>Laminar Shear Stress</i>
MA	<i>Mesenteric Arteries</i>
MAPK	<i>Mitogen Associated Protein Kinase</i>
MCP1	<i>Monocyte Chemotactic Protein 1</i>
MCU	<i>Mitochondrial Calcium Uniporter</i>
Mdivi-1	<i>Mitochondrial division inhibitor 1</i>
Mdv1	<i>Mitochondrial division protein 1</i>
MEK	<i>MAP2K</i>
Mff	<i>Mitochondrial Fission Factor 1</i>
Mfn-1	<i>Mitofusin 1</i>
Mfn-2	<i>Mitofusin 2</i>
MiD49	<i>Mitochondrial Dynamics 49 protein</i>
MiD51	<i>Mitochondrial Dynamics 51 protein</i>
MIEF1	<i>Mitochondrial Elongation Factor 1</i>
MLC	<i>Myosin Light Chain</i>
MM	<i>Mitochondrial Matrix</i>
mNCX	<i>Na (Sodium) Ca (Calcium) Exchanger</i>
MnSOD	<i>Manganese Superoxide Dismutase</i>
mPTP	<i>Mitochondrial Permeability Transition Pore</i>

mRNA	<i>Massenger Ribosome Nucleic Acid</i>
MS-1	<i>Mile Sven 1</i>
NADH,H ⁺	<i>Nicotinamide Adenine Dinucleotide</i>
NF	<i>Normal Flow</i>
NfκB	<i>Nuclear Factor kappa-light-chain-enhancer of activated B cells</i>
NO	<i>Nitric Oxide</i>
NOS	<i>Nitric Oxide Synthase</i>
NOS3	<i>Nitric Oxide Synthase 3</i>
NpY	<i>neuropeptide Y</i>
OH	<i>Hydroxyl Group</i>
OMA	<i>Metalloendopeptidase mitochondrial peptidase</i>
OMM	<i>Outer Mitochondrial Membrane</i>
ONOO ⁻	<i>peroxynitrite</i>
OPA1	<i>Optic Atrophy Protein 1</i>
OSS	<i>Oscillatory Shear Stress</i>
OVX	<i>Ovariectomized</i>
oxLDL	<i>Oxidized Low Density Lipoprotein</i>
OXPHOS	<i>Oxidative Phosphorylation</i>
P2Y	<i>Purinergic 2 Receptor</i>
PCR	<i>Polymerase Chain Reaction</i>
PDGF	<i>Platelet Derived Growth Factor</i>
PECAM-1	<i>Platelet Endothelial Cell Adhesion Molecule 1</i>
PGD2	<i>Prostaglandin D2</i>
PGE2	<i>Prostaglandin E2</i>
PGH2	<i>Prostaglandin H2</i>
PGI2	<i>Prostaglandin I 2</i>
PGI-2	<i>Prostaglandin I2</i>
PGI-3	<i>Prostaglandin I3</i>
Phe	<i>Phenylephrine</i>
PI3K	<i>Phosphatidylinositol-3-Kinase</i>
PKA	<i>Protein Kinase A</i>
PKC	<i>Protein Kinase C</i>
pTEN	<i>Phosphatase and Tensin Homolog</i>
q-RtPCR	<i>Quantitative Real Time Polymerase Chain Reaction</i>

RAS	<i>Renin-Angiotensin System</i>
RBP4	<i>Retinol Binding Protein 4</i>
RNA	<i>Ribonucleic Acid</i>
RNA	<i>Ribonucleic Acid</i>
ROS	<i>Reactive Oxygen Species</i>
Rpm	<i>Revolution Per Minute</i>
RyR	<i>Purinergic Y Receptors</i>
siCTL	<i>Silencing Control</i>
siDnm1l	<i>Silencing Mitochondrial division dynamin 1</i>
siOPA1	<i>Silencing Optic Atrophy Protein 1</i>
SUMO	<i>SUMOylation</i>
T2Dm	<i>Type 2 Diabetes mellitus</i>
ThAo	<i>Thoracic Aorta</i>
Thbd	<i>Thrombomodulin</i>
TNF- α	<i>Tumor Necrosis Factor alpha</i>
TP	<i>Thromboxane-Prostanoid</i>
tPA	<i>Tissue Plasminogen Activator</i>
TxA2	<i>Thromboxane A2</i>
uPA	<i>Urokinase</i>
VDAC	<i>Voltage Dependent Anion Channel</i>
VEGF	<i>Vascular Endothelial Growth Factor</i>
vWF	<i>Von Willebrand Factor</i>
WT	<i>Wild Type</i>
YAP	<i>Yes Associated Protein</i>
YME1L	<i>ATP-dependent metalloprotease</i>
Ψ_m	<i>Mitochondrial membrane potential</i>

Chapter 1: *The structure and functions of the vascular system*

The vascular system is a closed circulatory system that consists of arteries, veins and interconnecting capillaries. It functions in gas transportation, nutrients, metabolites and cells, but also it provides paracrine-signaling molecules to adjacent tissues. In general, it functions in maintaining an appropriate environment for the survival and the optimal functioning of cells.

Conversely, the lymphatic system is blind-ended and unidirectional, and comprises lymphatic vessels, lymph nodes and associated lymphoid organs. It belongs to the vascular system since it is coupled to the blood vasculature to return extravasated fluid and macromolecules to the blood circulation. Its function is to participate in fat absorption, tissue cholesterol clearance and immune cell tracking (provides an entry site for tissue-resident immune cells into the tissues) (Aspelund Aleksanteri et al. 2016).

The blood vessels are organized into tree-like structure. They transport blood in two distinct circuits: the systemic and the pulmonary circulation. In the systemic circulation, large arteries carry blood rich in oxygen away from the heart and branch into smaller arterioles that feed into extensive capillary networks. On the other hand, venules drain capillaries and converge into larger veins to return oxygen-poor blood to the heart. However, in the pulmonary circulation, pulmonary arteries transport oxygen-depleted blood to the lungs for gas exchange. Then, pulmonary veins return the oxygen-rich blood from the lungs to the heart. Despite the morphological and functional difference of these vessel types, arteries and veins share certain common characteristics (Figure 1).

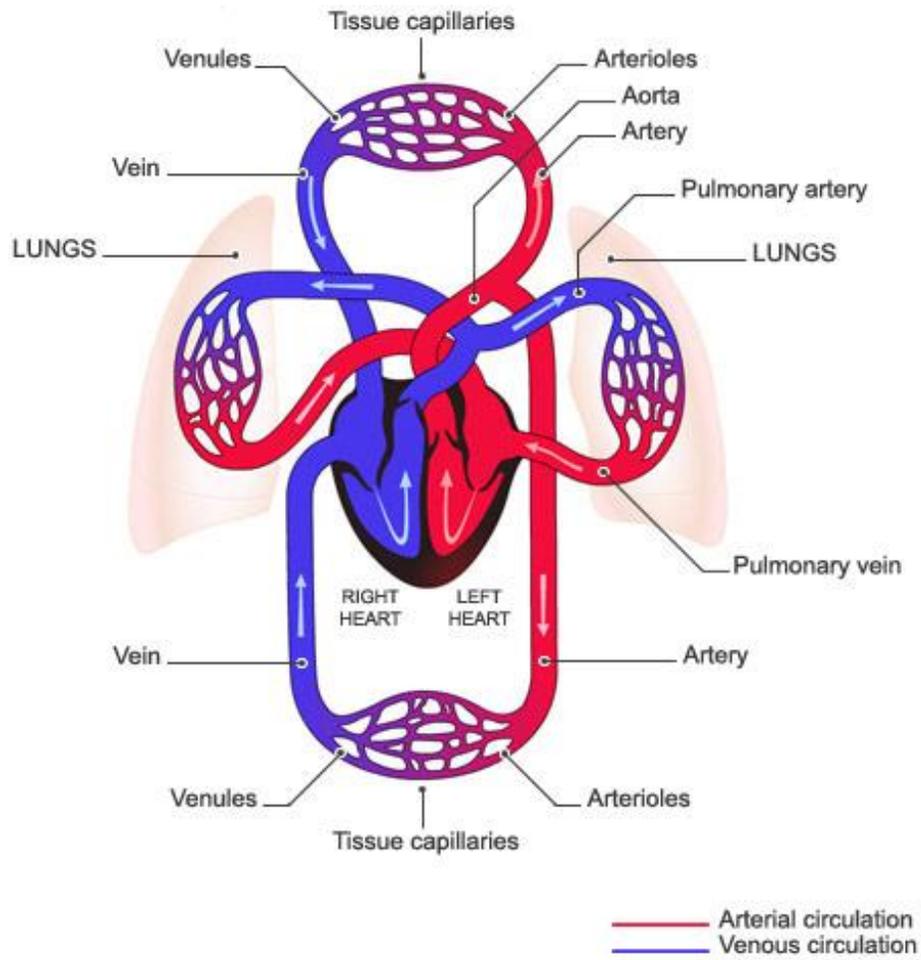


Figure 1 Schematic representation for the vascular system

(source: <http://www.urgo.co.uk/260-the-venous-system-within-the-cardiovascular-system>)

1. Arteries structure

There are three types of arteries:

Elastic or conductive arteries are the large arteries located near the heart. They have a very developed tunica media, rich in elastic fibers, collagen and muscle fibers. Its low resistance to the blood flow is a main character for this type of arteries. These correspond to large arteries such as the aorta, pulmonary arteries, carotids whose role is to conduct blood from the heart to smaller arteries. This type of arteries contains a high percentage of elastic fibers in their wall (about 40% of the total composition of the vessel wall), which allows them to support the blood pressure at the exit of the heart.

The **muscular or distributing arteries** are medium arteries whose media is richer in smooth muscle but less in elastic fibers than the conducting arteries. This gives them a great capacity for vasoconstriction and vasodilation, which allows them to regulate blood flow to the tissues.

The **arterioles** are the smallest arteries, located in the tissues just before the capillaries. Arterioles just located near the arteries have a similar composition to arteries. While for those located near the capillaries, the tunics are limited to one endothelial cells ring surrounded by some smooth muscle cells layer. They provide blood flow between the arteries and the capillaries of a tissue depending on the activity of the organ concerned (Figure 2).

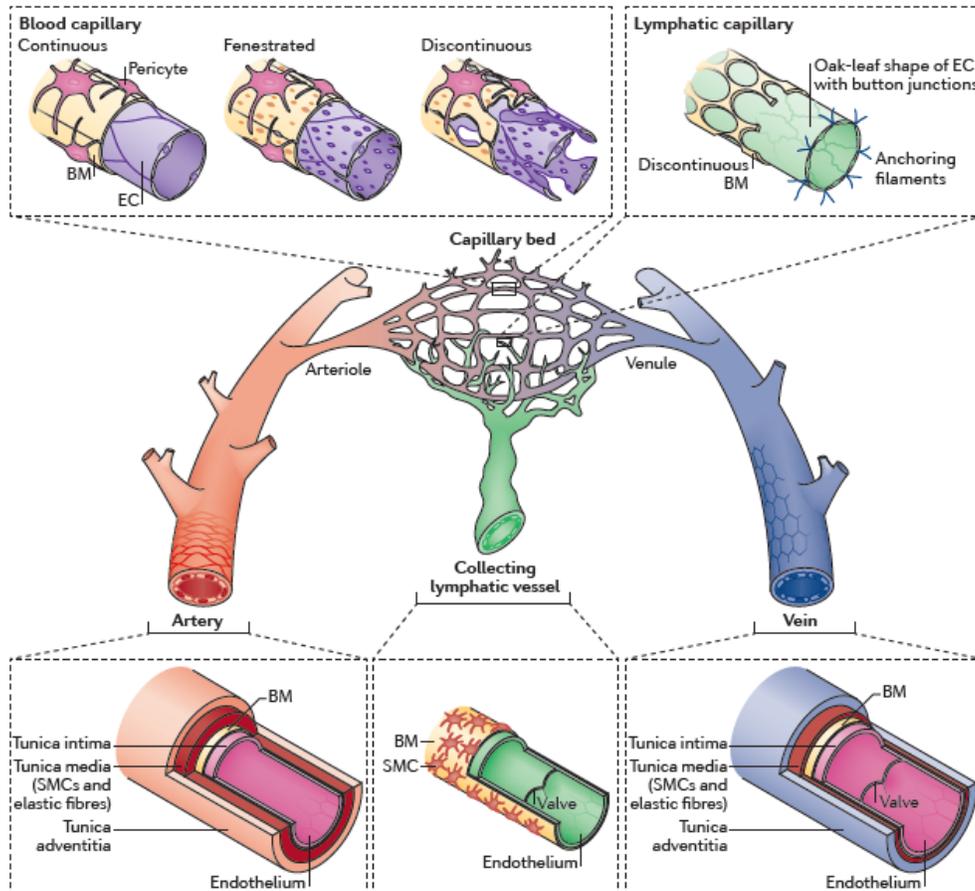


Figure 2 Organization of the vasculature (Potente and Mäkinen 2017)

The capillaries have a very small diameter, located around cells in the tissues between arterioles and venules. They only consist of a single layer of endothelial cells and a basement membrane, without a medium or external tunic. As they ensure the exchanges between the blood and the cells, the speed of flow in this microcirculation being only 8 mm per second. The sphincters between arterioles and capillaries distribute the blood mass as needed, if although at all times, only 5% of capillaries are supplied by arterioles. Then, venules are small veins formed by the union of several capillaries. They collect the blood from the capillaries and spill it into the veins. Venules, which are near the capillaries, consist of an endothelium and some smooth muscle cells. In contrast to those close to the veins, which have an outer coat similar to that of the veins.

The veins have a very large diameter and are made up of the same three tunics as the arteries but their relative thickness is different. They mainly contain collagen and few smooth muscle fibers. They provide the blood returns to the heart and forms a blood reservoir (Tortora, Derrickson, and Tortora 2009).

Therefore, vascular wall consists of three layers arranged concentrically from the endo-luminal cavity to the periphery of the artery: the intima, the media and the adventitia. Each of them plays a role in vascular pathophysiology.

1.1. Tunica Intima

Tunica intima is mainly constituted from a monolayer of endothelial cells (ECs) with a thin layer of connective tissue, the basal lamina, on which the ECs rest and interact with other cell layers. It is in direct contact with the circulating blood and therefore with metabolites, hormones, and all blood transporters. This layer is similar whatever the vascular territory but can however include structural modifications adapted to their functions. For example, the fenestrations at the level of the glomerulus of the kidneys, where glomerular endothelium plays the role of vascular permeability and glomerular filtration, in contrast to the cerebral arteries endothelium which acts as a strong barrier. Until recently, the endothelium was seen simply as the border between blood and tissues. The endothelium is now defined as an organ by its own, which plays a fundamental role in various physiological processes, and its dysfunction leads to an imbalance in the vascular homeostasis thus favoring the development of vascular pathologies (Potente and Mäkinen 2017).

The ECs are flattened about 0.5 μm thick, 100 μm long and 10 μm wide. Whatever the diameter of the wall, there is always a single layer of endothelial cells. The ECs are diamond-shaped and their juxtaposition constitutes a mosaic. Their major axis is elongated in the direction of blood flow and this orientation is determined by the shear stress applied to their surface. Histologically, the endothelium constitutes 1% of the total body mass and covers an area estimated at 28 m for the arteries and 280 m for the microcirculation. They have many organelles characteristic of a secretory cell. For example, the Weibel Palade bodies that contain the Von Willebrand factor are specific to the ECs. Historically, the endothelium was considered an inert, non-thrombogenic cell barrier until 1960. The first endothelial cultures were put in place in 1973, followed by the discovery of the essential role of the endothelium in relaxing the arteries (EDRF) and the discovery showing that EDRF is nitrogen monoxide (NO) produced by the ECs won the Nobel prize in medicine in 1998 (Robert Furchgott, Ferid Murad, Louis J. Ignarro) (Raju 2000).

1.2. Tunica Media

Tunica media is the central and usually the thickest layer in the arterial walls. It consists of layers of smooth muscle cells (SMCs) supported by connective tissue, which is mainly composed of elastic fibers and collagen. It is limited by the internal elastic laminae (IEL) and external elastic laminae (EEL), and thick fibers of elastin pierced with fenestrations. The middle coat (tunica media) is distinguished from the inner

coat (tunica intima) by its color and by the transverse arrangement of its cells. Besides, intercellular spaces between ECs are large enough to allow the bidirectional passage of substances and cells.

The contraction and relaxation of the smooth muscle cells regulate the diameter of the vascular lumen, as a result, decrease and increase blood flow. This vascular reactivity is regulated by several factors such as the nervous system, hormones and local chemicals.

The media is a layer, which is very variable according to the different vascular territories, and the presence and organization of both elastic fibers and SMCs vary according to the function of the vessels. The arteries can be classified according to the constitution of the media. Taking into account the relative proportion of the constituents of this matrix, three main types of arteries are thus distinguished:

- Elastic arteries, close to the heart, of large diameter, (aorta, large supra-aortic arterial trunks, iliac arteries) have a tunica media rich in elastic fibers and collagen, which ensure the arterial compliance needed to dump the blood during the systole and use their collagen content to resist the flow pressure.
- The muscular arteries, far from the heart, of medium or small diameter ($2 \text{ mm} \leq d \leq 5 \text{ mm}$), the most numerous (femoral, splenic, renal arteries) where the poverty in elastic fibers is characteristic of the media. The layer of SMCs as well as the ECs ensures the vasomotor properties of this arterial type. The media contains exclusively SMCs and extracellular constituents: elastic fibers, elastin fibrils, collagen bundles and fibrils, proteoglycans.
- Resistance arteries, in the microcirculation, these are vessels of small diameter and functions in the resistance to the blood flow. Usually, they are the end-points of the arteries. They have a media rich in SMCs, where they contract and produce more cells in response to blood flow that causes the distension of the matrix elements.

There is no net limit between the three types of arteries, which form a continuum changing progressively from elastic arteries to muscular arteries and to resistance arteries.

SMCs are elongated cells (30-200 μm long and 5 μm in diameter), mononuclear and surrounded by a basal lamina. Although the dimensions of the vascular smooth muscle cell (vSMCs) narrow toward their ends, end-to-end junctions coupling smooth muscle cells are complex and contain a significant number of membrane invaginations to provide increased surface area for both mechanical tight junctions and electrical coupling via gap junctions. These connections allow the transmission of the tensile force from one cell to another.

vSMCs contain a significant number of invaginations along the plasma membrane, called caveolae, which act to increase the cellular surface. These specialized invaginations further provide a unique plasma membrane environment, which enables clustering of specific groups of ion channels and receptors, which are important in cellular signal transduction.

vSMCs synthesize the components of the vascular wall (collagen type I, II, III and IV, elastin and proteoglycans). Proteoglycans form a highly hydrated network in which cells and fibrous proteins are immersed. These cells with the elastic limits form lamellar units (Wolinsky and Glagov 1967). The number of lamellar units is proportional to the diameter of the vessel, and gradually increases with weight and size in different animals. This organization in lamellar structure only exists in elastic arteries, where the notion of lamellar unit was completed in 1985 by that of a more precise muscular-elastic sheet: it is a group of cells enveloped by a matrix made up of a basal lamina and collagen fibrils, all wrapped in an elastic fiber fabric (Clark and Glagov 1976). In arterioles, there are generally no elastic lamellae, and the layers of vSMCs are much less numerous but always arranged in the same way (1 to 2 layers of vSMCs) (Fernando and Movat 1964). In the venules and certain veins, the vSMCs and the elastic fibers are rather organized longitudinally, along the axis of the vessel. In the veins, the two types of organizations coexist with more or less importance depending on the type of veins (Levy and Tedgui 2007).

Under normal physiological conditions, vSMCs have a contractile phenotype, characterized by a contractile apparatus composed of filaments and dense bodies (Figure 3). The filaments are of three kinds: myosin (15 to 19 nm in diameter), actin and structural proteins, such as vimentin and desmin (filaments of intermediate size of 10 nm). The thick myosin filaments are arranged in groups of 3 to 5 filaments, and are surrounded by a number of fine actin filaments, regularly organized and grouped in bundles respecting an actin/myosin ratio of approximately 15: 1, depending on artery type (Devine and Somlyo 1971).

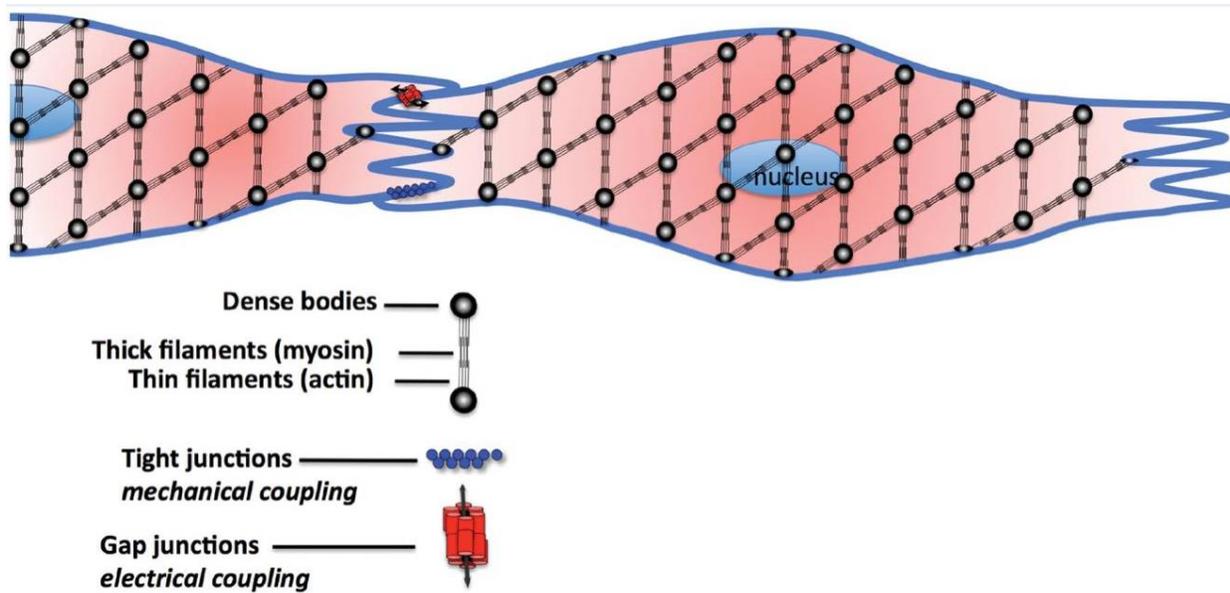


Figure 3 Smooth muscle cell structure (Wilson 2011)

Consequently, vSMCs play critical roles in cardiovascular diseases such as stroke and atherosclerosis and in metabolic disorders such as diabetes and obesity.

1.3. Tunica adventitia

The tunica adventitia is the most external layer of the vascular wall surrounding the tunica media. It contains connective tissue composed of elastic fibers and collagen; the vasa vasorum that supply with blood the wall of the large blood vessels; and also the nerve endings. Collagen serves in anchoring the blood vessels to nearby organs in order to maintain vascular stability.

In contrast to tunica media, its organization is almost the same regardless of the type of vessel. However, in the veins, very often the media and the adventitia are difficult to distinguish (absence of EEL in veins). Vascular cells must receive nutrients and oxygen (O_2) and repel gaseous and metabolic wastes. For that, most of the vascular cells directly carry out their exchanges with the circulating blood. Nevertheless, for large diameter vessels, the nourishment of the cells distant from the lumen of the vessel and the circulating blood is ensured through a microvascular system: the vasa vasorum. It is present in all arteries with more than 29 lamellar units (Wolinsky and Glagov 1967). The vasa vasorum can provide nutrients to vascular cells and also provide a number of mediators and hormones to the SMCs of the large arteries (Wolinsky Harvey 1970). This arteriolar and capillary network can be used in pathological situations of the vascular wall. Thus, at the level of atherosclerotic plaques at advanced stages, one can distinguish an angiogenesis of the cells feeding the vessels of the adventitia towards the intima within the plaque for nourishing the cells trapped into the lesions.

In addition, blood vessels are innervated by nerve fibers, the afferents of which extends to the limit of the media and the adventitia.

2. Factors controlling arteries functioning

2.1. Neuronal control

While most of the vasomotor nerves are noradrenergic, cholinergic nerve endings also exist. The nerve endings release neuromodulators, which act directly on the SMCs.

Briefly, these sympathetic nerve endings of blood vessels release mainly norepinephrine. The binding of this molecule to the adrenergic receptor α_1 of SMCs leads to an increase in intracellular calcium through the release of Ca^{2+} from the endoplasmic reticulum and the entry of extracellular Ca^{2+} . This increase in intracellular $[\text{Ca}^{2+}]$ induces the constriction of SMCs. In vessels containing several layers of SMCs, the outermost cells receive this sympathetic signal. Then, the innermost cells of the media contract through diffusion of norepinephrine and cell-to-cell communication through gap junctions (Thomas 2011).

In addition, the vascular sympathetic nerves can also contain neuropeptide Y (NPY) and ATP, which are released as co-transmitters. Both can induce vasoconstriction by activating Y1 receptors or P2X purinergic receptors, respectively, and cause an increase in intracellular $[\text{Ca}^{2+}]$ leading also to contraction (Pablo Huidobro-Toro and Verónica Donoso 2004).

In proportion to its metabolic needs, one of the main principles of the circulatory functions is the ability of each tissue or organ to control its blood flow.

2.2. Humoral Factors

The sympathetic nervous system also exerts indirect effects on the vessels by activation of the humoral systems including the release of epinephrine and norepinephrine by the adrenal medulla and the renin-angiotensin system (RAS). Blood pressure and vascular homeostasis are regulated mainly by renin-angiotensin system. The RAS system is the most important endocrine system controlling the regulation of blood pressure. It plays a major role in a large number of pathophysiological regulations, in particular the regulation of vascular tone. It is also involved in the pathophysiology of most cardiovascular diseases. It is well established that the RAS is not only a circulating hormonal system, but that it exists in a wide variety of tissues (intrinsic RAS), in addition to the intracellular generation of angiotensin II, which also makes the RAS as a paracrine and an intracrine system (Kumar, Singh, and Baker 2007).

Indeed, all angiotensin peptides originate from the same precursor, angiotensinogen (AGT), through its sequential cleavages by the classic enzymes, renin and angiotensin converting enzyme (ACE), or enzymes

of alternative pathways. AGT, from a structural point of view, belongs to the family of serine protease inhibitors (serpins) although it is not known to have enzyme inhibiting activity, it gives rise to a spectrum of angiotensin peptides with angiotensin Ang II being the major effector peptide that regulates blood pressure and sodium/water homeostasis (C. Wu et al. 2011).

Renin, secreted in the kidneys, is responsible for the conversion of AGT, produced by the liver, to angiotensin I. Angiotensin I is then converted into angiotensin II (Ang II) by the angiotensin converting enzyme (ACE) which is secreted, mainly by the endothelium of the lungs, and epithelial kidney cells. Angiotensin II binds to its transmembrane G-protein-coupled receptors angiotensin II type 1 receptor (ATR1) and type 2 receptor (ATR2). These two receptors functions oppositely after the binding of Ang II to it. Besides, an alteration in the proper balance in RAS induces potential harmful consequences on the cardiovascular system by initiating vascular damage (van Thiel et al. 2015).

AT1R are expressed mainly in the adrenal glands, SMCs, kidneys, heart and the brain. All of the known regulatory effects of angiotensin II on blood pressure have been attributed to the AT1R. Therefore, their stimulation is responsible for most of the physiological and pathophysiological effects of angiotensin II: vasoconstriction, stimulation of aldosterone and vasopressin synthesis, decrease in renal blood flow, sodium reabsorption in the renal tubules, hypertrophy of smooth and myocardial muscle cells and angiogenesis. In addition, angiotensin II, via the AT1R, directly causes cell growth, regulates the expression of genes of various bioactive substances (vasoactive hormones, growth factors, components of the extracellular matrix, cytokines, etc.), and activates several intracellular signaling cascades (MAPKs, tyrosine kinases, various transcription factors, etc.) in myocytes and cardiac fibroblasts, ECs, SMCs, and renal cells. However, AT2R are expressed ubiquitously in fetal tissues, suggesting a role for this receptor in the development of the fetus. On the other hand, they are much less abundant in adult tissues, although they are still expressed in the adrenal medulla, the uterus, the ovaries, the vascular endothelium, and specific areas of the brain. AT2R, therefore, seem to be involved in the control of proliferation and cell differentiation, angiogenesis, wound healing, tissue regeneration, and even apoptosis (Figure 4) (van Thiel et al. 2015; Verdecchia et al. 2008).

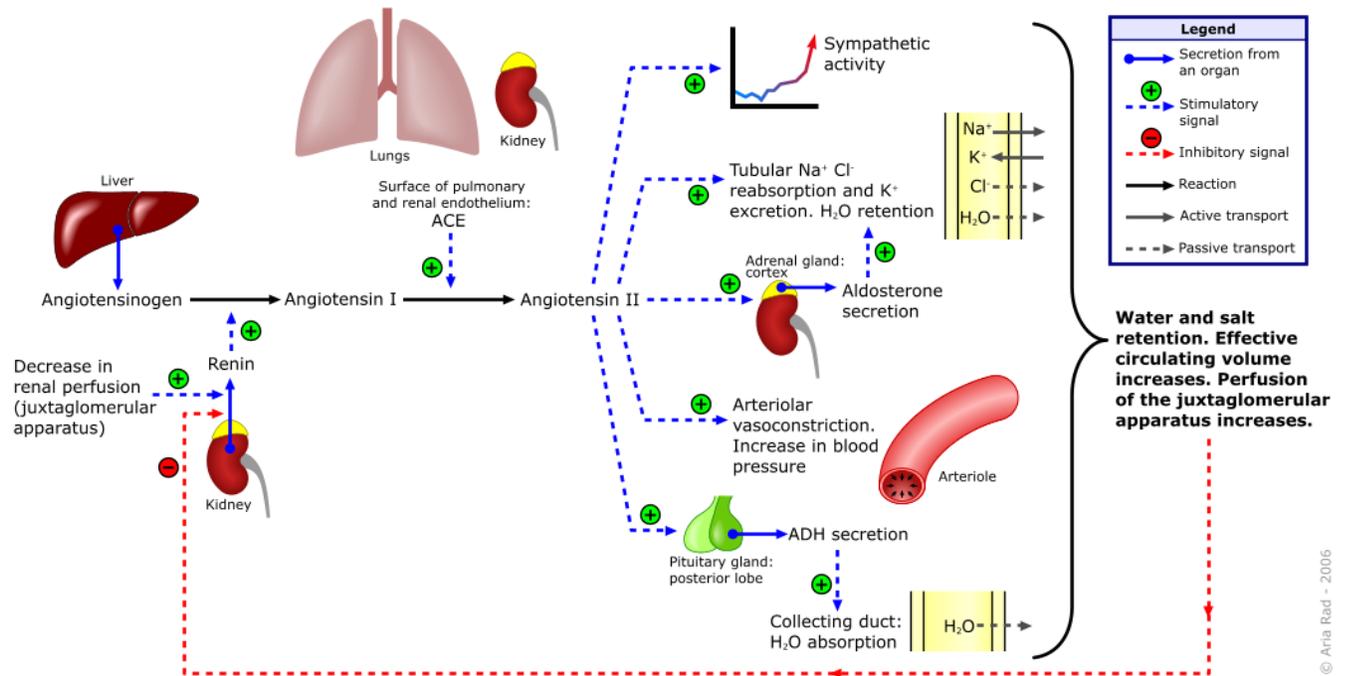


Figure 4 Schematic diagram showing the Renin Angiotensin System regulatory mechanisms (de Almeida and Coimbra 2019)

2.3. Local Chemicals

Besides the autonomous nervous system, vascular smooth muscle cells tone is controlled by the neighboring endothelial cells. Through the release of different vasorelaxing and vasoconstricting factors, endothelial cells are capable to interact with smooth muscle cells in order to maintain vascular homeostasis. The endothelium plays its role in vascular homeostasis by the secretion of many vasoactive agents among which nitrogen monoxide (NO), prostacyclin (PGI₂), hyperpolarizing factors (EDHF) and vasoconstrictive factors like endothelin 1 (ET-1), thromboxane (TXA₂) are the most important.

2.3.1. Endothelium derived vasorelaxing factors (EDRF)

Nitric Oxide

Nitric oxide Nitric oxide (NO) is a gaseous element produced from arginine. NO is formed by the enzyme NOS (Nitric Oxide Synthase), which converts the amino acid L-arginine to L-citrullin and NO. There are three NOS isoforms: type I or neural NOS (nNOS), which produces NO as a neuronal messenger; type II or inductive NOS (iNOS), which is expressed only in cells exposed to inflammatory mediators; and type III constitutive or endothelial NOS (eNOS) which produces nitric oxide in the vascular endothelium. NO has been shown to play an important role in maintaining a basic vasodilatation of blood vessels.

NO is a determining regulator involved in the control of the vascular tone, but also exerts an anti-inflammatory, anti-apoptotic, anti-mitogenic and antithrombotic properties (Harrison et al. 2006). This effect is regulated either by a transcriptional increase in the gene coding for eNOS, or by a post-transcriptional modification of its phosphorylation.

The inactive eNOS is linked to the protein caveolin and is found in small invaginations in the cell membrane called the caveolae. Several factors such as bradykinin (BK), acetylcholine (Ach), adenosine tri-phosphate (ATP), and adenosine di-phosphate (ADP) can stimulate eNOS by releasing Ca^{2+} from the endoplasmic reticulum. Activation of the eNOS starts by its coupling with Ca^{2+} / Calmodulin complex. The activated eNOS then converts L-arginine to NO and citrulin. Once synthesized, NO in the ECs diffuses to the adjacent SMCs, where it exerts its physiological effect of vasorelaxation (Figure 5). In SMCs, NO activates second messengers, especially Guanylyl cyclase (GC), this enzyme becomes activated and increases the conversion of GTP to cGMP, which decreases smooth muscle tension. cGMP can in turn activate ion channels, protein kinases, and phosphodiesterases (Ignarro 1992). However, in response to shear stress, ECs are able to release NO more plenty than to other agonists that elevate intracellular calcium levels. An increase in shear stress have been shown to regulate vascular homeostasis through the increase in eNOS phosphorylation and NO production (Corson Marshall A. et al. 1996).

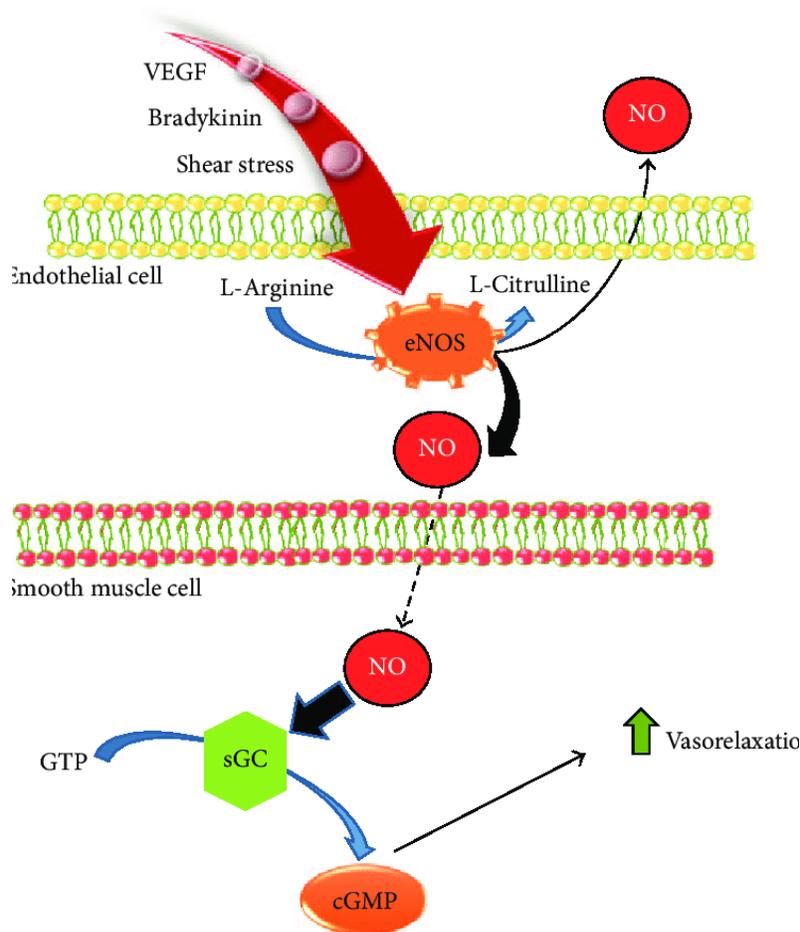


Figure 5 Effect of the release of NO after direct stimulation of endothelial cells (Forte et al. 2016).

The normal function of eNOS requires the dimerization of the enzyme, the presence of the substrate L-arginine, and the essential cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄). An increase in blood flow and blood pressure promotes the phosphorylation (phospho-Tyrosine-14) of caveolin-1 (cav-1), which leads to the dissociation of eNOS from cav-1. Free eNOS associates with calmodulin (CaM), which in turn allows the activation of eNOS by its phosphorylation at serine 1177 at the luminal surface level. However, phosphorylation at serine 635 have been reported to play an important role in enhancing the enzymatic activity of eNOS (Iring et al. 2019). This phosphorylation of this enzyme at serine 635 is slower than the phosphorylation at serine 1177 (Boo et al. 2002). Rizzo et al have shown that 90% of the eNOS detected are co-localized in caveoli when the ECs have been subjected to the flow (Rizzo et al. 2003). In addition, Yu et al have demonstrated that active phosphorylation of eNOS was reduced in carotids from mice lacking caveolin-1 (cav1^{-/-} mice). This effect was independent of an alteration in the localization of eNOS, these results suggest that in cav1^{-/-} mice, flow is unable to activate the upstream kinases for eNOS activation. However, these effects could be restored by expressing a transgene of caveolin-I specifically at the

endothelium level (Yu et al. 2006). Thus, caveolin-1, in addition to its ability to directly modulate allosterically the activity of eNOS, can also act as a scaffold protein to group proteins involved in flow-dependent signaling in structures called signalosomes.

In cardiovascular diseases such as hypertension, there is an overproduction of reactive oxygen-derived species (ROS). These superoxides react with NO to form peroxynitrite (ONOO⁻). The ONOO⁻ is able to oxidize the BH₄ cofactor very easily. The decrease in the level of BH₄ induces the production of O²⁻ by the eNOS. This process, called decoupling of the eNOS, contributes to endothelial dysfunction (Förstermann and Münzel 2006).

Prostaglandin I₂ (PGI₂)

Prostacyclin is a potent inhibitor of platelet activation and is produced by the concerted actions of cyclooxygenase and prostacyclin synthase. This potent inhibitor of platelet aggregation acts also as a powerful vasodilator. PGI₂ is synthesized in endothelial cells, smooth muscle cells and fibroblasts.

When hormones or physical forces activate cells, intracellular calcium increases and activates calmodulin, which in turn activates cytosolic forms of phospholipase A₂ (PLA₂). Activated PLA₂ cleaves arachidonic acid (AA) from the membrane phospholipids. AA detaching from phospholipids of the membranes is converted by cyclooxygenases to cyclic endoperoxides, and then PGI₂ through PGI₂ synthase. The predominant enzymes in the metabolism of arachidonic acid (AA) are the enzymes cyclooxygenases (COX) comprising two isoforms COX-1 and COX-2. Although COX-1 and COX-2 share 65% structural homology, the activity and expression of these enzymes are regulated differently, and they can function independently in the same cell type. These two enzymes are expressed in ECs and also in SMCs but at a lower level. COX-1 is expressed constitutively while COX-2 is expressed when tissue is damaged or exposed to inflammatory factors. COXs convert arachidonic acid (AA) to prostaglandin H₂ (PGH₂), which is then synthesized into various eicosanoids: prostacyclin (PGI₂), prostaglandin D₂ (PGD₂), prostaglandin E₂ (PGE₂), prostaglandin F₂α (PGF₂α) and thromboxane A₂ (TXA₂) by their synthetic enzyme.

PGI₂ is a prostanoid that acts in regulating vascular homeostasis. It is an unstable substance formed by the enzyme "prostacyclin synthase" (PGIS). In ECs, this enzyme is highly expressed, and is closely associated with COX-1. Several studies have more recently shown that COX-2 also participates in the synthesis of PGI₂. PGI₂ binds to its specific IP receptors, located on vascular SMCs and platelets. The binding of PGI₂ to platelet receptors leads to the inhibition of platelet aggregation. The binding of PGI₂ to the IP receptors of SMCs activates adenylate cyclase AC, which induces the synthesis of cyclic adenosine monophosphate (cAMP). cAMP then activates protein kinase A (PKA), which allows relaxation of SMCs in the same way as NO. The role of prostacyclin as a mediator of endothelium-dependent relaxation has been shown since

in certain vascular territories; its effect cannot be observed when the other pathways have been inhibited. In vascular diseases, PGI₂ can play a compensatory role in the event of a decrease in the bioactivity of NO. In addition, in many vascular areas, PGI₂ can also react as a hyperpolarizing substance derived from the endothelium (Figure 6).

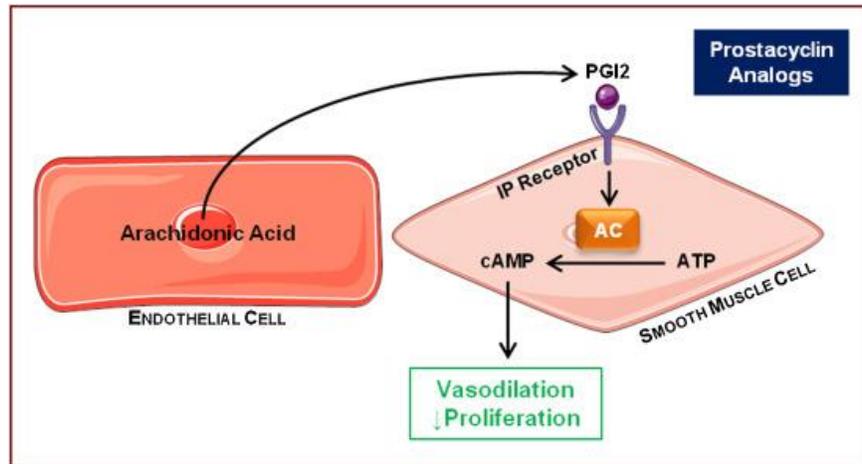


Figure 6 PGI₂ signaling pathway for vasorelaxation (Santos-Ribeiro et al. 2016)

2.3.2. Endothelium derived hyperpolarizing factor (EDHF)

The residual component of endothelium-dependent relaxations observed in the presence of inhibitors of eNOS and COX, is now attributed to endothelium-dependent smooth muscle cells hyperpolarization (Griffith 2004). Such responses are critical to the regulation of tissue perfusion due to their predominance in small vessels and the resistant vasculature (Berman et al. 2002). In response to various stimuli such as bradykinin and acetylcholine, ECs synthesize and release vasoactive factors, which can induce hyperpolarization in SMCs.

NO and PGI₂ are two already well-known substances. However, when these two molecules are inhibited, hyperpolarization still occurs, suggesting the involvement of one or more hyperpolarizing factors. It has been termed endothelium-derived hyperpolarizing factor (EDHF). In blood vessels from various species including humans, endothelium-dependent relaxations are partially or very resistant to inhibitors of NO synthase and cyclooxygenase and are observed without an increase in the intracellular level of cyclic nucleotides in the vascular smooth muscle cells. The evidence that hyperpolarization was suppressed by Apamine and Charybdotoxin, the specific inhibitors of the Small-conductance calcium-activated potassium channels (SK channels) and calcium activated potassium channels in ECs, suggests the implication of these two types of channels in this phenomenon (Félétou and Vanhoutte 1999).

The existence of EDHF as a diffusible substance has been demonstrated under bioassay conditions whereby the source of EDHF was either native vascular segments or cultured endothelial cells. The identification of EDHF may allow a better understanding of its physiological and pathophysiological role(s).

In recent years, a much simpler theory suggested that the EDHF phenomenon might involve the electrotonic spread of hyperpolarization from the endothelium to smooth muscle cells via myo-endothelial gap junctions. There is growing evidence showing that agonist stimulated endothelium-derived hyperpolarization may be conducted electro-tonically to subjacent smooth muscle cells with the resulting hyperpolarization causing reductions in $[Ca^{2+}]_i$ and vasorelaxation (Griffith 2004) (Griffith, Chaytor, and Edwards 2004). Activation of endothelial receptors induces a subsequent increase in $[Ca^{2+}]_i$, which then causes the K^+ to exit the cell. SMCs respond to the change in extracellular K^+ and also releases K^+ , causing hyperpolarization. The change in membrane potential of SMC reduces $[Ca^{2+}]_i$, resulting in vasodilation (Figure 6).

EDHF-type relaxations have been defined as a distinct dilatation mechanism on the basis that they are insensitive to eNOS and/or COX inhibitors. Several lines of evidence implied that EDHF is the main vasorelaxant mechanism in small size arteries where NO bioavailability is limited (Busse et al. 2002).

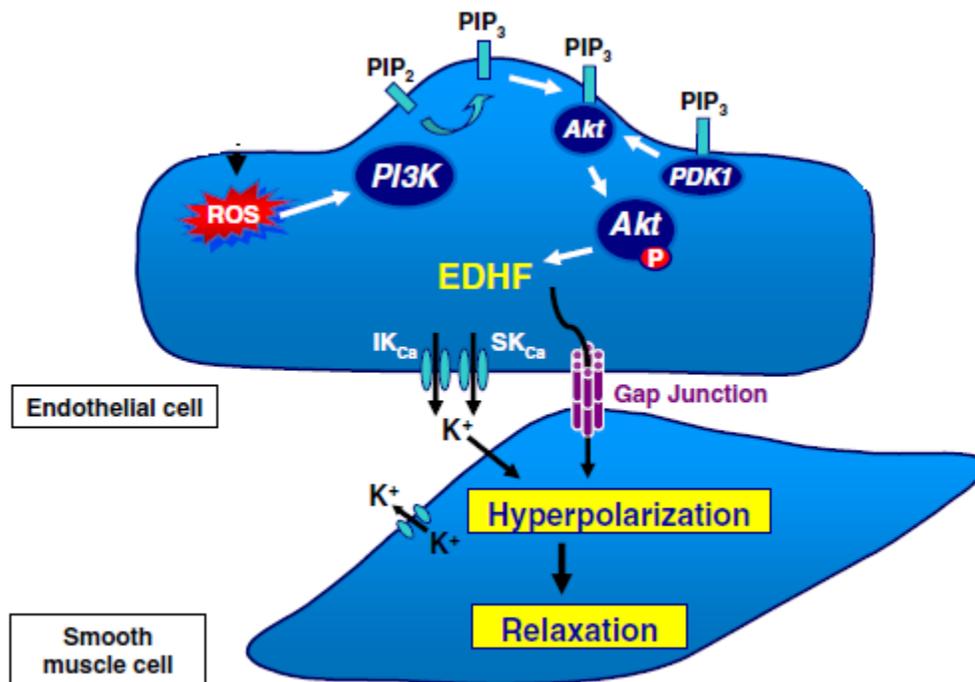


Figure 7 EDHF signaling pathway leading to vasorelaxation (Schini-Kerth et al. 2010) (modified)

2.4. Endothelium derived vasoconstriction factors (EDCF)

These are factors mainly derived from arachidonic acid through cyclo-oxygenase pathway. These pathway products have been shown to act as vasoconstrictors in kidney and systemic circulation, the fact that endothelial cells could also mediate vasoconstriction by interacting with SMCs.

Endothelin-1 (ET-1)

Endothelin is a potent vasoconstrictive peptide that was first isolated from porcine endothelial cells in 1988 (Yanagisawa et al. 1988) and initially shown to play a role in inflammatory pain (Ferreira, Romitelli, and de Nucci 1989). There are three isoforms of endothelin peptides, ET-1, 2, and 3, of which ET-1 is the only one produced by the endothelium.

ET-1 is a 21 amino acid peptide synthesized from its precursor, pre-pro ET-1, via a proteolytic cleavage facilitated by the metalloproteinase, endothelin-converting enzyme (ECE) (Grant, Loizidou, and Taylor 2003, 1). The release of this molecule is stimulated by inflammatory factors such as IL-1, TNF- α and is reduced by NO, and PGI₂. Endothelins exert their physiological effect through binding of two receptors, endothelin A (ETAR) and B (ETBR). They are G-protein coupled transmembrane receptors found on many different types of tissue. ET-1 receptors have been identified on both SMCs (ETA and ETB₂) and ECs (ETB₁). ETAR interacts preferentially with G_q and G_s while ETBR interacts preferentially with G_i and G_q (Figure 8).

The binary function of endothelin leads to divergent actions in the vasculature, where ETAR mediates vasoconstriction and ETBR mediates vasodilatation, depending on their expression on different vascular cells (Pan et al. 2008). When ET-1 binds to ETA receptors, the Ca²⁺ channels of SMCs open, allowing Ca²⁺ to enter the cell, this causes vasoconstriction. On the contrary, the binding of ET-1 to the ETB₁ receptors of the endothelium causes vasodilation by inducing the release of NO and PGI₂ (Lüscher T F et al. 1992).

On the other hand, ET-1 also causes inflammation and proliferation of SMCs. Binding of ET-1 to ETA receptors activates macrophages, but also increases the adhesion of neutrophils to the vascular wall, free radicals ending in an endothelial dysfunction (Sandoo et al. 2010).

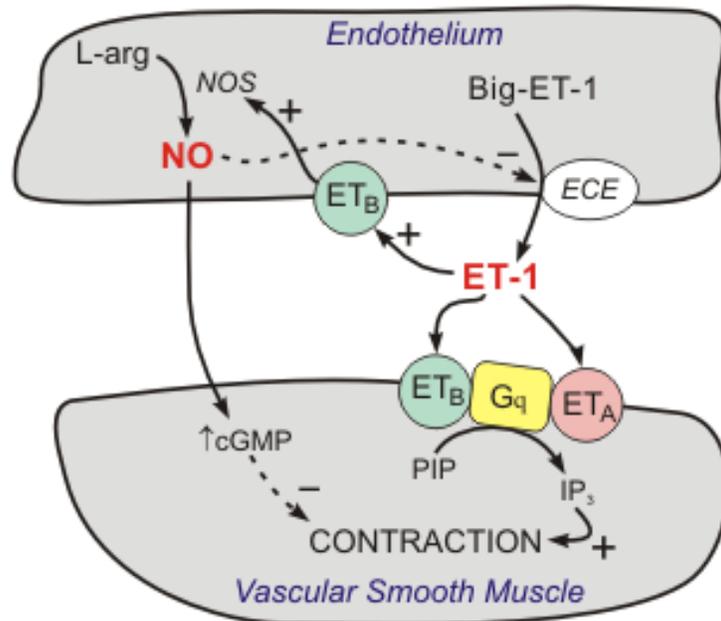


Figure 8 Schematic diagram for the action of ET-1 on both, ECs and SMCs (Richard E. Klabunde 2014)

Thromboxane A₂ (TxA₂)

TxA₂ is synthesized from the hydrolysis of arachidonic acid released from phospholipids by phospholipase A₂ (PLA₂). Newly liberated arachidonic acid is converted to PGH₂ by cyclo-oxygenase 1 (COX-1) and then PGH₂ is converted into TxA₂ through TxA₂-synthase. Once formed it can easily diffuse across the plasma membrane and activate other platelets that are in close proximity. TxA₂ acts through two G-protein coupled receptors on platelets and SMCs, thromboxane-prostanoid (TP α/β) receptors that are coupled to Gq and G12/G13 families (Henriksen Ruth Ann, Samokhin Gennady P., and Tracy Paula B. 1997).

Activation of TP induces several physiological responses, including platelet adhesion and aggregation, contraction, proliferation of SMCs, and activation of endothelial inflammation. Similar to ADP signaling, TxA₂ signaling involves the activation of PLC β isoforms by Gq to increase the formation of IP₃ and DAG and therefore enabling Ca²⁺ mobilization and PKC activation. It is now well established that TxA₂ signaling initially drives a first wave of ADP release which promotes integrin activation and aggregation. In contrast, thrombin-induced aggregation does not require secondary mediators. The TP receptor isoforms are also coupled to G12/G13, which has been linked to platelet shape change. Platelet shape change has been shown to involve the interaction between myosin and actin through the phosphorylation of myosin light chain (MLC). The phosphorylation of MLC is regulated by MLC kinase and myosin phosphatase. Platelet stimulation by TxA₂ results in the phosphorylation of the MLC and inactivation of myosin phosphatase in a Rho-kinase dependent manner (Henriksen Ruth Ann, Samokhin Gennady P., and Tracy Paula B. 1997).

Activation of TP induces several physiological responses, including platelet adhesion and aggregation, contraction, proliferation of SMCs, and activation of endothelial inflammation. TP deficient mice have normal blood pressure but tend to bleed. In addition, the suppression of TP decreases the hypertension induced by angiotensin II as well as the effect of L-NAME, and the associated cardiac hypertrophy. As TXA2 and PGI2 are produced by COX, the balance of these two molecules helps to maintain the homeostasis of the vessel (Ricciotti Emanuela and FitzGerald Garret A. 2011).

Reactive oxygen species (ROS)

ROS are classified as both free radicals such as the superoxide anion (O_2^-), the hydroxyl radical (OH \cdot), peroxynitrite (ONOO \cdot) and non-radicals such as hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). In the vessel, ROS plays an important role in the regulation of different physiological and pathophysiological processes where they are produced by ECs but also by SMCs and fibroblasts. NADPH oxidase (NOX) has been shown to be one of the major sources of ROS. Other sources of ROS in vessels have been described including mitochondrial electron transport chain (ETC), xanthine oxidase, decoupling of eNOS (Touyz and Schiffrin 2004).

For many years, ROS was considered only as deleterious molecules because of their ability to cause oxidative damage to different cellular molecules and structures, and their clear involvement in the induction and progression of many pathologies as well as in the aging process. However, in the last years it is becoming evident that only uncontrolled high ROS levels are dangerous and, on the contrary, controlled low ROS levels are normally produced in all cells, contributing to different physiological processes through the regulation of different molecules acting in the cellular pathways (Covarrubias et al. 2008). In particular, H_2O_2 is a good candidate to mediate cellular signaling, thanks to its characteristics: half-life relatively long and high diffusion properties. It has also been noticed that the specific targets of the ROS signaling are located close to the ROS generating systems, favoring ROS regulative functions (Reczek and Chandel 2015).

ROS can interact with each other, but also with all types of cell components: proteins, lipids, carbohydrates, and DNA. These effects depend on their own reactivity, their preferential affinity for a particular substrate, and their quantity and their ability to diffuse. Members of the NOX family are enzymes that produce superoxide anions by transferring electrons from NADPH to oxygen molecules. Unlike phagocytic NADPH oxidase which is activated only during stimulation, vascular NADPH oxidases are constitutively active and produce intracellular O_2^- in a slow and sustained manner and act as intracellular signaling molecules (Rodrigo, González, and Paoletto 2011).

In addition, mitochondria are also a major source of ROS. During oxidative phosphorylation in the mitochondria, the flow of electrons can accidentally cause an electron transfer to the oxygen molecule, which will give the superoxide anions as a byproduct during ATP synthesis. Another source of ROS, xanthine oxidase is mainly expressed in the endothelium and can be activated by angiotensin II. It catalyzes the oxidation of hypoxanthine or xanthine during the metabolism of the purines. During this process, xanthine oxidase reduces oxygen leading to the formation of H₂O₂ and O²⁻. Besides, the deficiency or oxidation of L-arginine and tetrahydrobiopterin (BH₄), the two essential cofactors of eNOS, leads to a decoupling of the eNOS, which produces ROS instead of NO. The decoupling of eNOS contributes significantly to the production of cellular ROS (Schulz, Gori, and Münzel 2011).

In contrast, there is an antioxidant enzyme system, protecting the cell from oxidative stress. Superoxide dimutases (SOD) play a major role in the defense of the cell. The three SOD isoforms have been identified: cytosolic SOD (CuZn-SOD or SOD-1), mitochondrial SOD (Mn-SOD or SOD-2) and extracellular SOD (ecSOD or SOD-3). They are responsible for converting O₂⁻ to H₂O₂. H₂O₂ is then converted to H₂O by catalase and glutathione peroxidase (GPx). Catalase is present mainly in peroxisomes and contains a ferric ion molecule at its active site, although GPx is a peroxidase, which is abundantly present in both the cytosol, and the mitochondria of mammalian cells (Figure 9) (Yung et al. 2006).

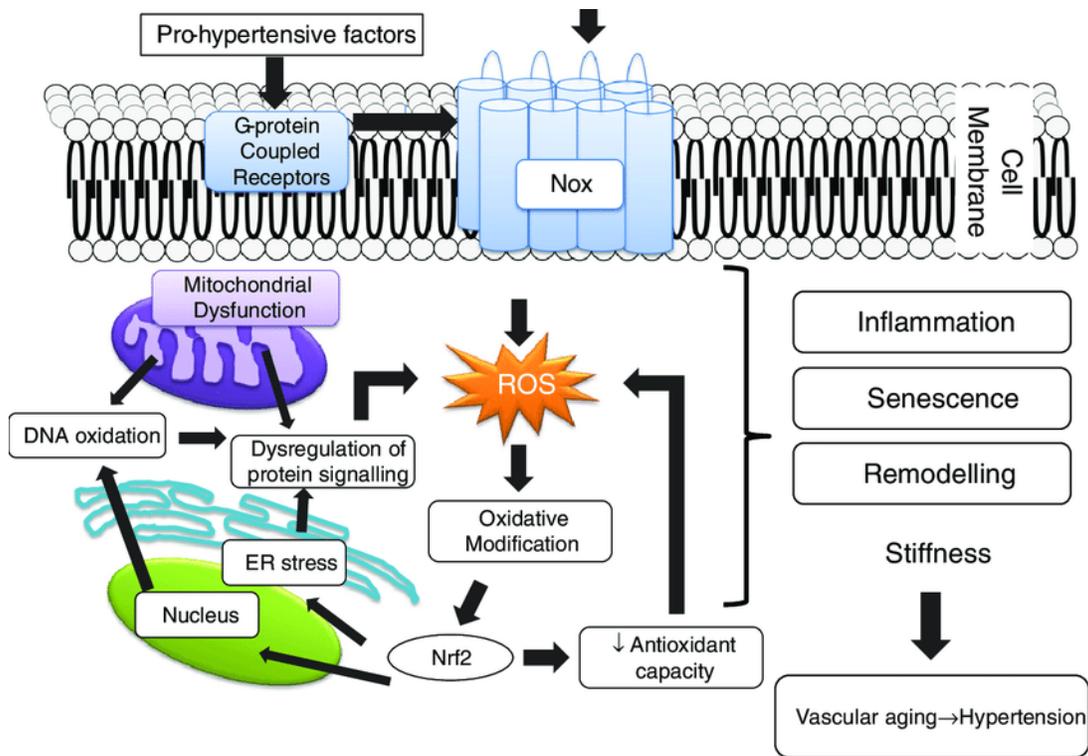


Figure 9 Vascular remodeling through ROS released in response to different pathological pathways (Harvey, Montezano, and Touyz 2015)

The action of different enzymes generating ROS after the stimulation of different factors could activate intracellular signaling pathways in both ECs and SMCs. In ECs, NOX activation produces cytosolic and mitochondrial superoxides that could activate physiological signaling pathways and/or interact with NO to produce ONOO. The latter interacts with BH4 to decrease its presence for the synthesis of NO through eNOS. eNOS, in turn, becomes uncoupled and becomes pathological by producing superoxide inhibiting its dilatory effect. On the other hand, SMCs phenotype is changed by ROS production leading to a change in cell growth and apoptosis resulting in hypertrophy, hyperplasia, alteration in extracellular matrix, and the migration of these cells. All of these changes will lead to a change in the vascular tone.

Chapter 2:
The
Endothelium

1. Physiology of the endothelium

The vascular endothelium is not a simple anatomical barrier between the circulating blood and the smooth muscle cells. It plays an important role in the regulation of various physiological parameters such as hemostasis, transport of angiogenesis metabolites, and control of vascular tone. Whatever the vessel wall diameter, there is always a single layer of endothelial cells. Histologically, the endothelium constitutes 1% of the total body mass and covers an estimated area of 28m² of the arterial area and of 280 m² of the microcirculation area (Augustin, Kozian, and Johnson 1994). The endothelial cells are very heterogeneous in phenotype according to their localization in the vascular tree. In general, endothelial cells have a flattened shape with 2µm thickness. They also share common characteristics with secreting cells.

The morphological heterogeneity of the endothelium can be explained by the heterogeneity of capillary and arterial structures. The capillary endothelium is very permeable with pores ranging from 70 nm to 3 µm. In small capillaries, a single endothelial cell covers the internal wall of the vessel forming a tube in which blood circulates. Capillary endothelial cells are particularly rich in caveolae and their membrane structures are rich in cholesterol, glycosphingolipids/sphingomyelin and proteins including caveolin, that participate along with other capillary elements in the transportation of large molecules through the wall. However, arterial endothelial cells present a squamous organization on the arterial wall and they show a weak permeability with few caveolae. Moreover, the exact endothelial cell physiology in the arteries essentially depends on the local shear stress applied.

The endothelium is not only a semi-permeable barrier but also a real functional tissue with several roles. Whether it belongs to the peripheral or cerebral circulation, to the macro or microcirculation, the endothelium regulates a multitude of functions through the synthesis and secretion of multiple factors activating different signaling pathways that regulates arterial tone and structure, maintains homeostasis, and modulates leukocyte diapedesis.

2. Arterial tone regulation

Arterial tone regulation is the alteration in the contraction or relaxation state of the muscular layer of the arteries. Different mechanisms regulate the balance between the two arterial states of vasoconstriction and vasodilation. The signals allowing a contraction or a relaxation of smooth muscle layer can come from either the tunica adventitia or the tunica intima. Although, signals coming from the adventitia are mainly vasoconstrictory signals including catecholamine and Ang II signaling cascades, the signals from the blood flow through the endothelial cells (tunica intima) are mainly vasodilatory and less often vasoconstrictory.

Different vasoactive molecules are released by the endothelium to regulate the vascular tone. Thus, it maintains vascular homeostasis by the secretion of many vasodilation agents including NO, PGI₂, and EDHF favoring relaxation of the vascular SMCs layer, and on the other hand, vasoconstriction agents among which ET-1, ROS and TXA-2 are the most important. These local chemicals produced by endothelial cells are detailed in chapter 1 detailing their origin, mode of action and their relative signaling cascade (refer to chapter 1, 2.3. Local Chemicals). An overview for the vasorelaxation and vasoconstriction factors acting in the vessels are shown in Figure 10 & Figure 11.

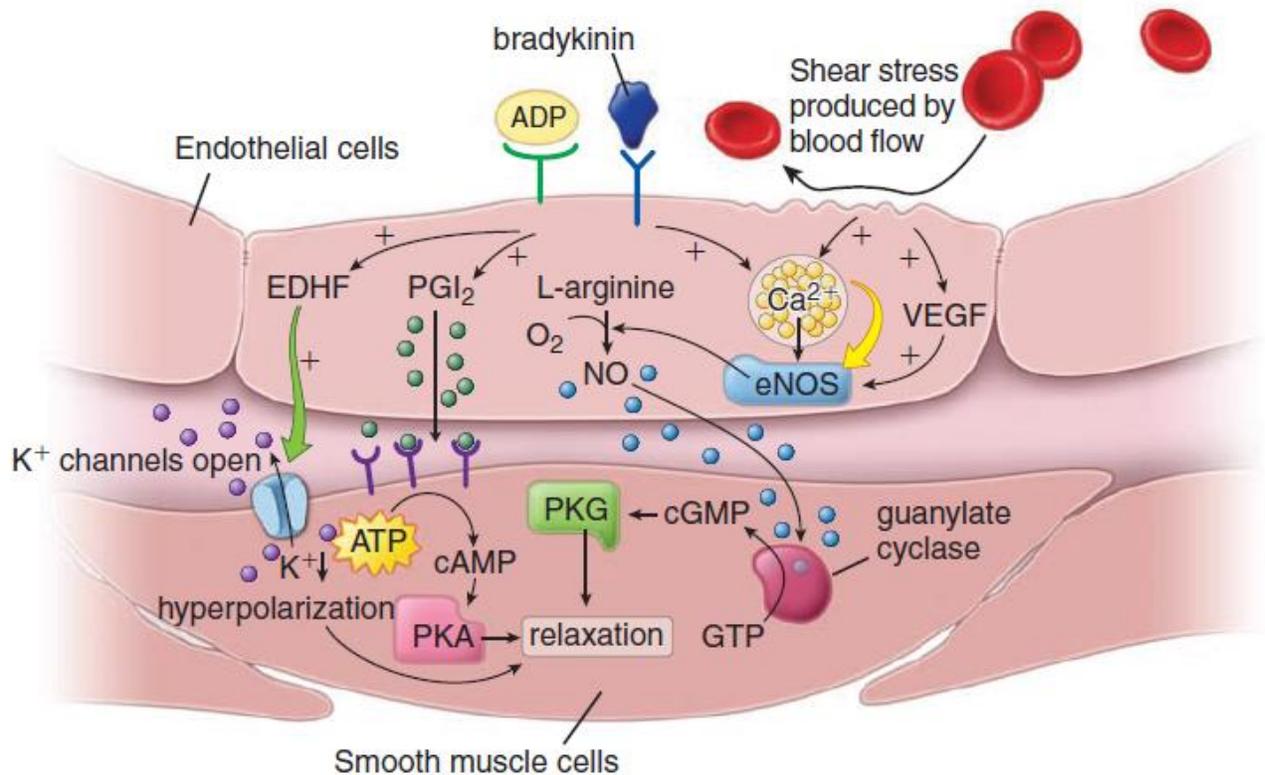


Figure 10 Vasorelaxation through endothelial signaling (Memorang n.d.)

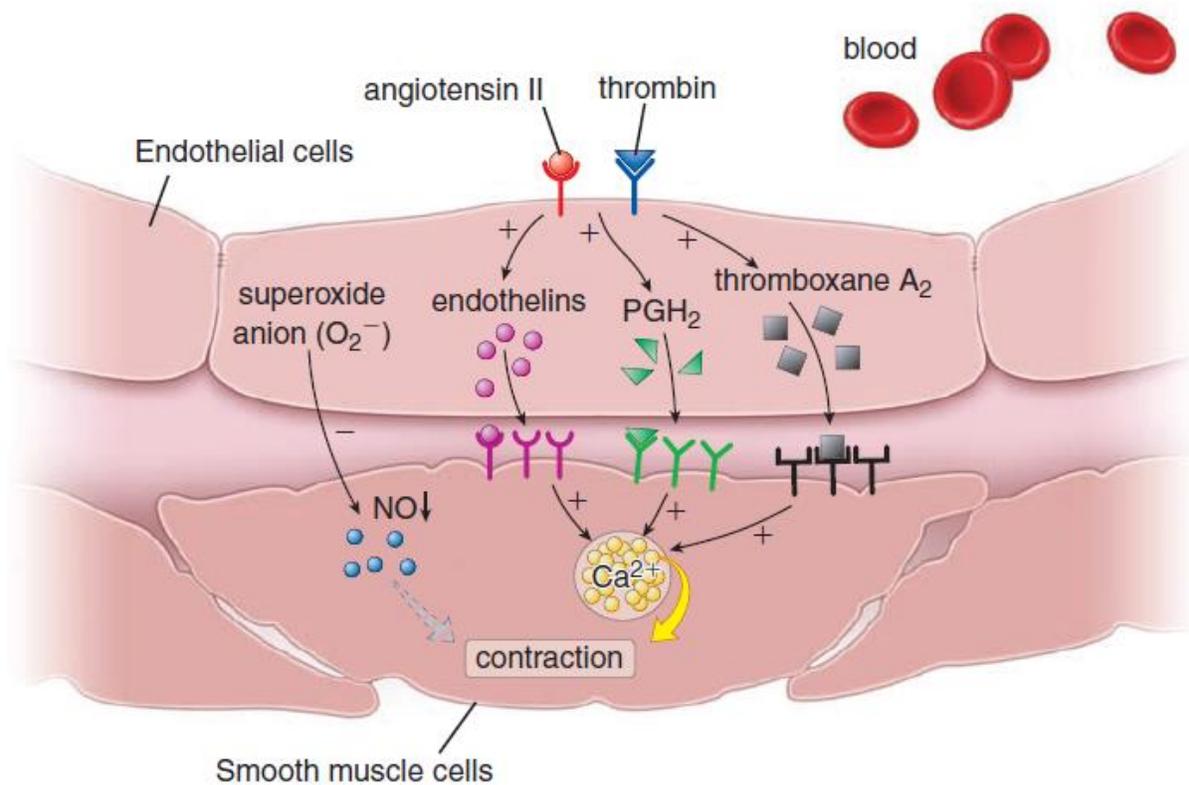


Figure 11 Vasoconstriction of SMCs via endothelial signaling (Memorang n.d.)

3. Ûm: Blood Hemostasis

Hemostasis can be defined as the set of mechanisms which contribute to maintaining the blood in a fluid state inside the vessels, namely to stop hemorrhages and prevent thrombosis. The endothelium plays an antithrombotic role, which keeps blood in fluidic state during circulation and consequently, prevents the formation of blood clots in the vessels. Therefore, if the vascular wall is damaged, the endothelium sets up the blood coagulation cascade.

Under normal conditions, the endothelium is endowed with anti-platelet, anti-thrombotic and pro-fibrinolytic properties. Endothelial cells achieve this function through its integrity and secretion of prostacyclin, which is a powerful inhibitor of aggregation, a vasodilator and anti-thrombotic factor.

During blood hemostasis, three stages are conventionally distinguished:

1. Primary hemostasis that closes the vascular injury with a "platelet plug".
2. Secondary hemostasis, coagulation consolidates this first thrombus by forming a network of fibrins imprisoning red blood cells (Blood clot formation).
3. Fibrinolysis allows the destruction of clots, or the limitation of their extension.

After injury, endothelial cells express an adhesion factor (Von Willebrand factor vWf), which will ensure the interaction between the platelets and the wall of the injured vessel. Then, these cells start to express prothrombinase complex, which is two tissue complexes that initiates the formation of thrombin that in turn converts fibrinogen to fibrin to produce a blood clot (Göbel et al. 2018). During the last step of fibrinolysis, the dissolution of the clot mainly accomplished by plasmin, an enzyme presents in the blood in the form of plasminogen. Two endothelial factors induce the transformation of plasminogen into plasmin: tPA (tissue plasminogen activator) and uPA (urokinase). tPA is weakly expressed in the basal state, its synthesis is increased several days after the onset of the lesion or after the secretion of other factors like adrenaline. Thus, the plasmin produced destroys the fibrin and dissociates the extracellular matrix allowing degradation of the clot (Figure 12).

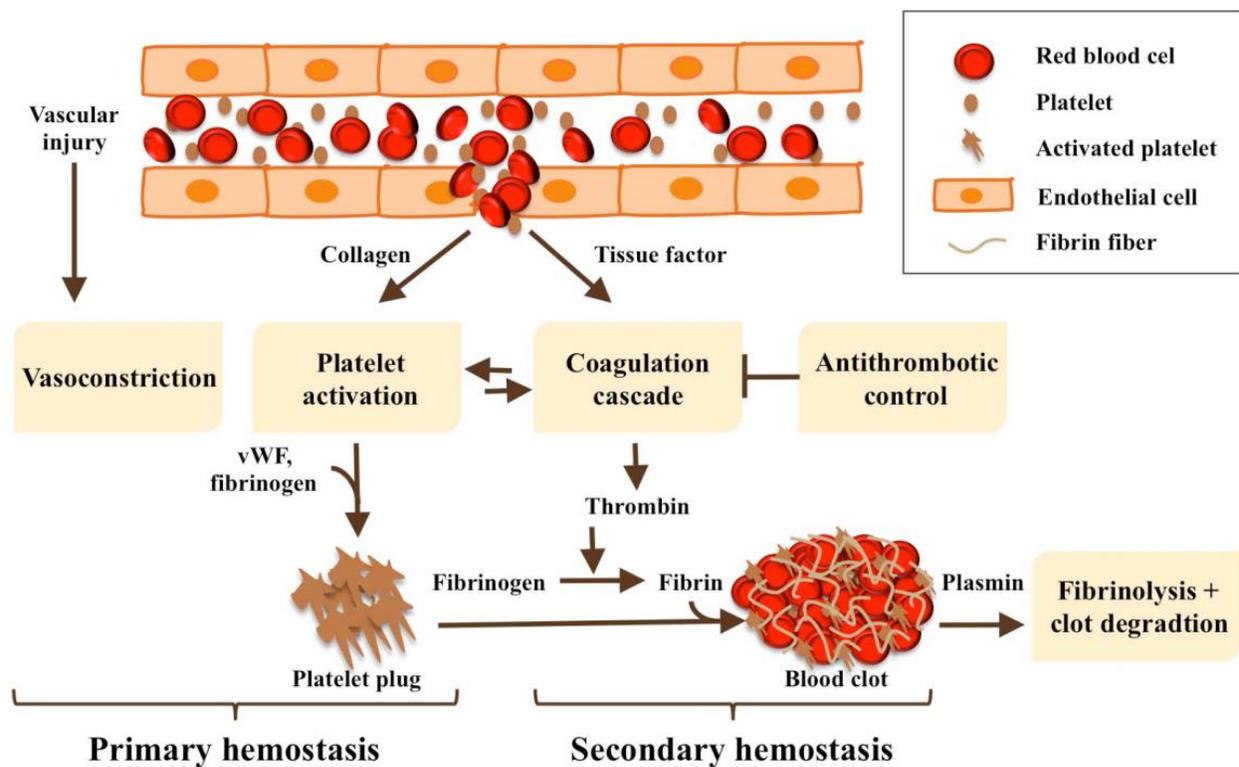


Figure 12 Mechanism of blood hemostasis (Labberton 2016)

Although, healthy endothelial cell also expresses thrombomodulin on its membrane that opposes the formation of thrombin, these cells express and activate the PAI-1 and PAI-2 proteins (plasminogen activator inhibitors), which make it possible to stop the action of tPA. Therefore, a fine regulation in the balance between the lysis and clotting takes place locally in the lesion sites.

4. Leukocyte extravasation

White blood cells (WBCs) are produced in the bone marrow and then migrate into the blood to reach the interstitial space. Leukocyte extravasation is the mechanism by which a leukocyte crosses the endothelial layer of a vessel to reach the site of inflammation. Endothelial cells control the WBCs transmigration by determining which of them will leave the blood stream and migrate into the surrounding tissues, known as WBCs sorting process. This mechanism taking place in response to pro-inflammatory signals will not only be perceived by leukocytes, but also activate the endothelium allowing the expression of adhesion molecules on their surface, including selectins, integrins and cell adhesion molecules (CAM). This will allow recognition between endothelial cells and leukocytes to activate rolling of the leukocytes on ECs through selectins, and adhesion of the leukocytes to the endothelium through integrins. As a result, leukocytes will be able to transmigrate through the endothelial barrier. Each step of these is under the specific control of one or a combination of the adhesion molecules expressed by the endothelium.

However, transmigration can be done in two ways: either the leukocyte extravasates at the level of cell junctions through local and temporary dissociation of the junction system (also known as, paracellular migration), or the leukocyte crosses the endothelial cell by opening a "hole" in the cytoplasm (also known as, transcellular migration). These two phenomena use common molecules on ECs like PECAM, ICAM1, CD99 but also specific molecules such as JAM-A, which is only involved during paracellular migration (Figure 13).

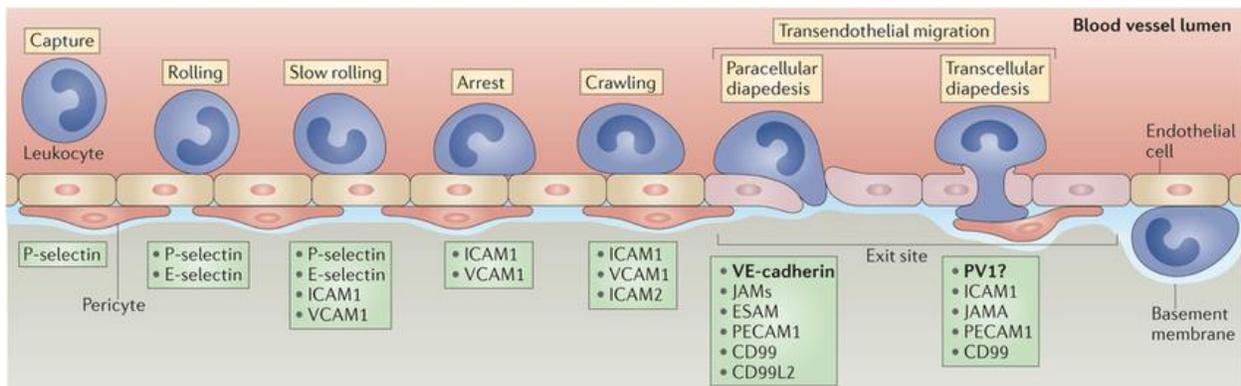


Figure 13 Mechanism for leukocyte extravasation process (C and N 2019)

5. The endothelium: mechanical sensor of the vessel

The vascular endothelium is permanently subjected to three types of mechanical stress by which the intensity of each varies according to the vascular bed type. These mechanical stress are due to the blood flow and hemodynamic forces exerted on the vessels. The three types are: the shear stress (τ), the hydrostatic pressure (p) and the periodic deformation or cyclic stretching (ϵ) (Charara et al. 1993).

As endothelial cells are located at the internal side of the arterial wall, they feel much more the blood flow than any other vascular cells and this is due to the frictional forces generated by flow. Thus, endothelial cells and smooth muscle cells are “equipped” to translate these external signals into intracellular messages allowing the vessels to respond to blood flow. This response is different according to the time of exposure to the stimuli.

In contrast, SMCs are only able to sense the hydrostatic pressure and stretching cycles. As my thesis is concerned mainly with shear stress as an internal mechanical stress of the vascular wall sensing the endothelium, I will focus more on the relation between shear stress and endothelium in the next paragraphs.

5.1. Endothelium and Shear Stress

Wherever flow occurs, shear stress exists. Shear stress is defined as the force per unit area created when a tangential force (blood flow) acts on a surface (the endothelium). It is determined by the flow rate (Q) and viscosity (μ) of the blood as well as by the radius (r) of the vessel according to Poiseuille's law (for a laminar flow):

$$T = 4 \mu Q / \pi r^2$$

In human, the average value of this stress, in a straight portion of the vessel is between 0.2 and 2 Pa, or 10 and 20 dyne/cm². This value may increase in some cases of vascular pathologies. In contrast, for a pulsatile flow the formula would take into account the frequency of the pulses.

The response of endothelial cells to mechanical stress has been extensively studied and variations in a large number of cellular functions have been reported (electrophysiological, biochemical, receptors, gene regulation, etc.). Besides, the effects of acute or chronic shearing had been reported, *in vitro*. Although, endothelial cell responses to shear stress are often simultaneous, endothelial responses could be classified according to reaction and exposure timing. For example, rapid and acute changes in membrane potential (seconds) and activation of biochemical cascades show a similar characteristic time. In this case, we see G protein activation, the release of NO, the mobilization of phosphoinoside derivatives, the release of intercellular Ca²⁺, the phosphorylation of cyclic nucleotides, etc. However, the structural changes such as the reorganization of actin filaments and cell morphology appear much later in the sequence of mechanotransduction events.

After a variation in the diameter of the vessel due to either vasoconstriction or vasodilation, a change in the level of shear stress with a modulation in the viscosity of the blood leads to a change in the shear force on endothelial cells. In blood, the major factor modulating viscosity is the concentration of red blood cells.

Under physiological conditions of strong shear stress, the endothelial cells adopt a particular morphology; they orient and align in the direction of flow. Whereas, under conditions of low shear stress (weak or oscillating flow), they lose this alignment capacity (Remuzzi et al. 1984).

Furthermore, the value of shear stress in the vessels will be dependent on the structure of the vessel. Indeed, the presence of irregularities, obstructions or curves will modify the local blood flow. Consequently, three type of flow could be distinguished depending on the arterial area subjected to flow (Figure 14) (Malek, Alper, and Izumo 1999):

- **Undisturbed laminar Flow:** In the linear areas of the vessels, the shear stress will be pulsatile due to the heartbeats but also, as the blood circulating in parallel lines with the inner endothelial cells layer, the flow is considered laminar. This type of stress is observed in the large arterial trunks, resistance arteries and arterioles.
- **Disturbed laminar flow:** In the areas of arterial bifurcations and curves, the flow becomes weaker (between 0 and 10 dyn/cm²).
- **Oscillatory turbulent flow:** At the level of the side branches, the flow also becomes oscillatory (bidirectional); the shear stress then vary around 0 dyn/cm².

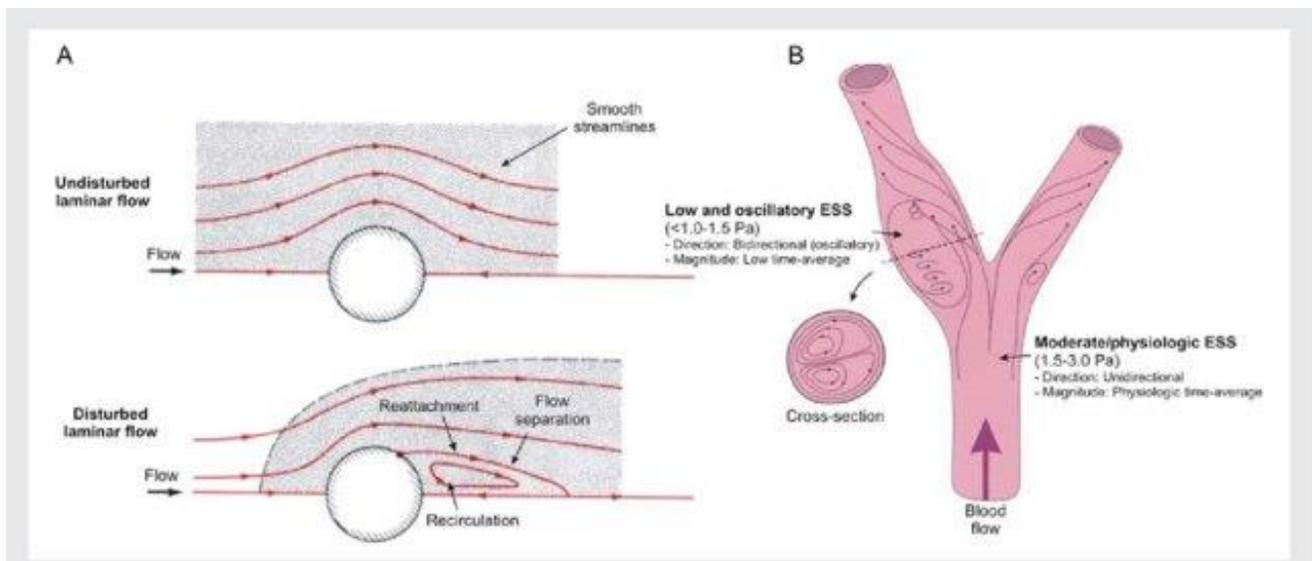


Figure 14 Different forms of flow with the change in the geometrical zones of the arteries (Wentzel et al. 2012)

Yet, for oscillatory rate flow measurement, the turbulent nature of the flow must be taken into account. This is defined by another parameter: Reynolds number. This number depends on the viscosity and speed of the fluid. For a given geometric conditions, the highly viscous fluid circulating weakly will retain a

laminar flow after an obstacle ($Re < 2000$). On the other hand, the faster and less viscous fluid is, the higher the turbulence after an obstacle will be ($Re > 2000$) (Figure 15) (Chatzizisis et al. 2007a).

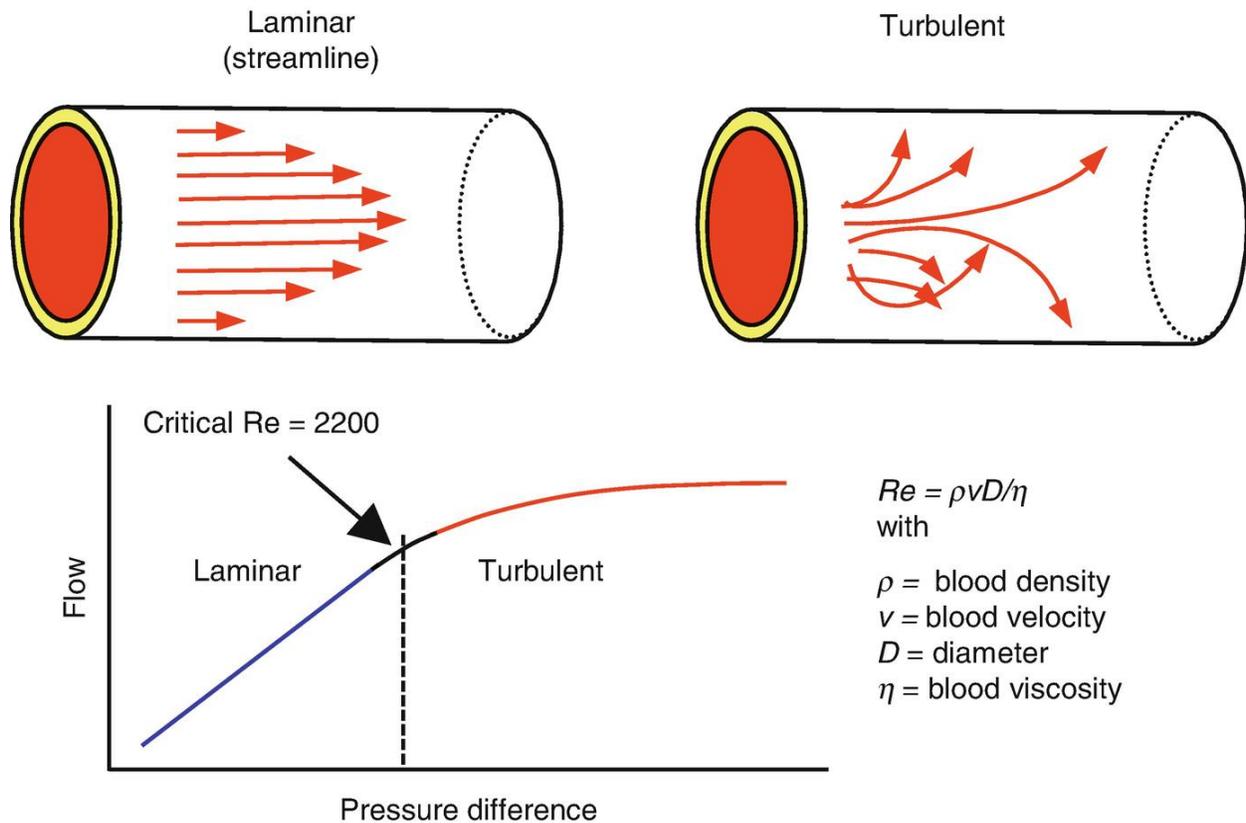


Figure 15 Blood flow dependence on Reynolds number (Westerhof et al. 2019)

5.2. Mechanosensitivity of shear stress

In response to shear stress, two type of ECs responses could be distinguished:

- An early response that is triggered within seconds of the onset of shear stress. It is linked to the mechanical force imposed by the flow.
- A late response corresponding to the adaptation of cells to a sustained flow.

The sense of shear stress by endothelial cells is called mechanosensitivity. For the endothelial cells to sense the force exerted by shear stress, they use different sensors on their surfaces. All the proteins whose conformation is directly regulated by tension could be classified as mechanosensitive proteins. Among these mechanosensitive proteins, we find in particular proteins whose mechanical stimuli allow the exposure of their peptide sequences to shear forces (Vogel 2006), allowing the recruitment or phosphorylation of other partners, and also the opening of certain ion channels (Martinac 2004). Other proteins acts after the change in the voltage generated by the cell due to exposure of their peptide sequences

to flow, whereas in the case of ion channels, the activity is regulated by the tension of membranes or by the attachment to junctions that ensure transmission of the mechanical signal to a biochemical one (Soattin et al. 2016). Although ion channels and fibronectin were the first elements to be described in the 2000s as mechanosensitive proteins, the list has since grown. Indeed, many other proteins also have the ability to regulate their conformation by the change in cell electrophysiology or even by conformational changes. Among these, we can find adhesion proteins and receptors such as integrins, junction molecules, GPCRs, VEGFR, caveoli, ion channels, and the glycocalyx (Figure 16).

Depending on the duration of elevated shear stress, some are activated in an early response (ion channels), while others will be more involved in late responses to shear stress (certain integrins). Likewise, the shear stress degree (weak or strong) will lead to different activations because the conformational change of proteins in response to mechanical stress may also affect the stability of protein interactions. This could be explained due to the fact that protein-protein interaction has a limited lifespan, and the application of a tensile force will decrease the duration of this interaction (Evans and Calderwood 2007).

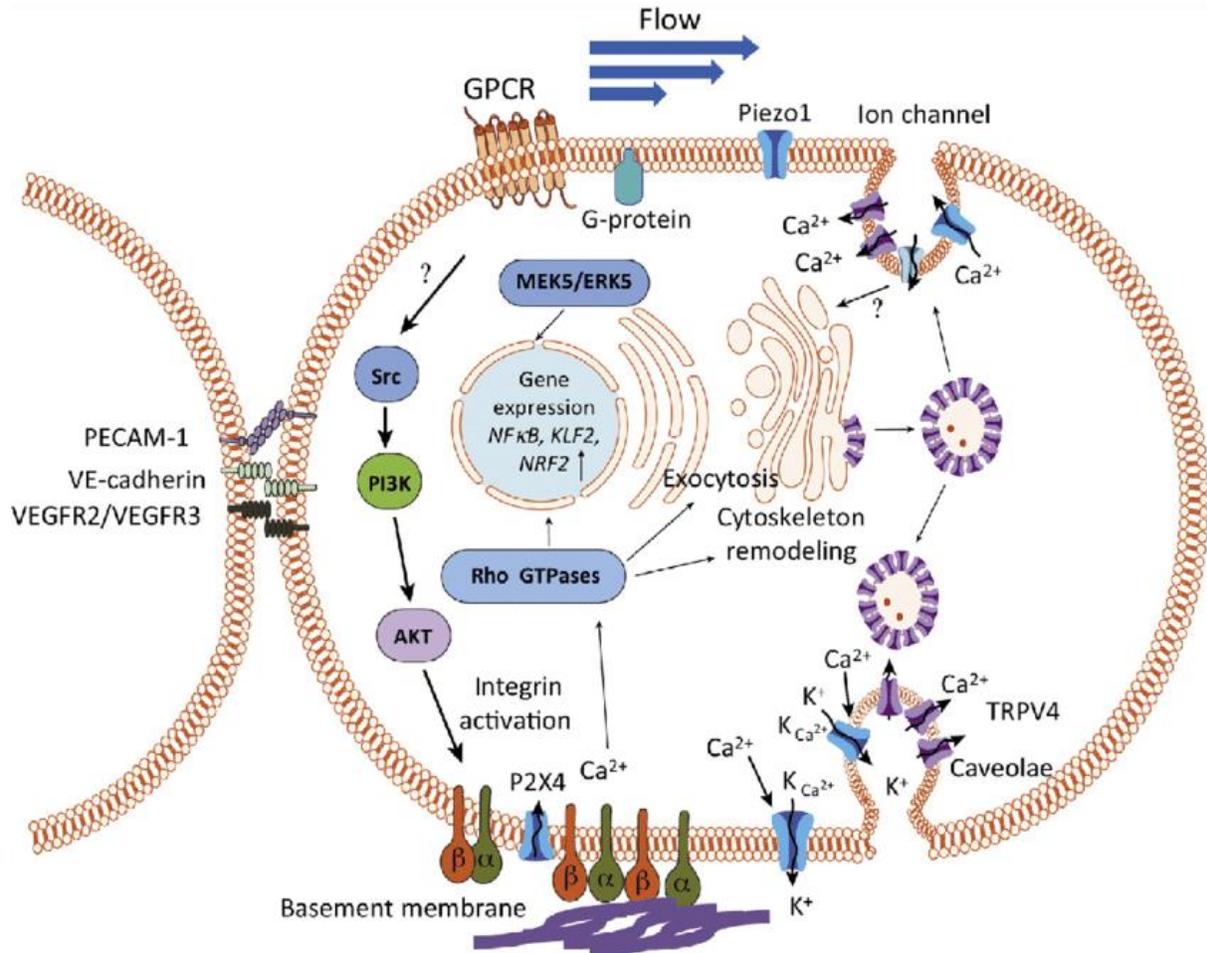


Figure 16 Mechanosensors of shear stress on endothelial cells (Baratchi et al. 2017)

On the contrary, the response of endothelial cells to shear stress has been studied invitro, also. Where they have distinguished two types of variations after ECs stimulus by flow Invitro ECs, in order to allow the cells to adapt to the modification of its environment (Ballermann et al. 1998)

- Early metabolic variation (SS < 6 hours): modification of the transcriptome and the proteome of genes having, in their promoter, a specific sequence called Shear Stress Responsive Element (SSRE). Certain genes, which are, involved in vaso-reactivity, like cell adhesion molecules, coagulation and growth factors, can modify their expression after shear exposure.
- Late morphological change (several days to weeks of SS): reorientation of cells, in the direction of flow, with reorganization of the EC cytoskeleton, and more particularly of the actin filaments.

5.3. Mechanotransduction of shear stress

Mechanotransduction is the transformation of a mechanical message into a biochemical message. The mechanosensors presented above are considered as an integral part of this phenomenon, which will lead to the activation of different signaling cascades and to the modulation of gene expression in ECs. Ultimately, different functions of these cells will be modulated. Endothelial mechanosensors can interact together, activate each other, act on common or different pathways, for a long-term or short-term period.

In response to high shear stress, several intracellular signaling transduction pathways are activated. Ionic channels activated by shear stress are one of the potent activators of PKC proteins that will rapidly increase the levels of ET-1 produced by ECs. ET-1 binds directly to its ET-B receptor leading to the liberation of NO as a second messenger signaling molecule to activate activator protein AP-1 in its c-jun part. Consequently, AP-1 regulates the expression of the genes involved in the response to shear stress (Morawietz et al. 2008). Noting that, PKB also participates in the activation of Akt by high shear stress (Go et al. 2001).

Moreover, more proteins could be activated by high shear stress on ECs, such as protein kinases C (PKC), focal adhesion kinases (FAK), small Rho proteins, phosphoinositide 3 kinases (PI3K), PYK2 tyrosine kinase protein, and “mitogen-associated protein kinases” (MAPK).

After stimulation of ECs by shear stress through integrins, an activation of the Ras/MAPK pathway is activated through the phosphorylation of FAK on tyrosine residue. SH2-domain containing proteins will be able to bind to FAK just after its phosphorylation and thus, Src binds to FAK and increases its phosphorylation at Tyr-925 creating a site for Grb2/Sos binding. These events will lead to the activation of Ras/MAPK by converting Ras from inactive GDP state to GTP active state. This signaling complex leads to the activation of ERK and JNK proteins in response to shear stress (S. Li et al. 1997). However, mechanotransduction that ends in MAPK pathway serves in many functions in endothelial cells, where it regulates proliferation, differentiation, mitosis, cell survival, apoptosis and gene expression as well as many other functions (Pearson et al. 2001). Clearly, GPCRs contribute to the activation of these MAPKs by ligand independent mechanotransduction manner. Shear stress leads to conformational change in the structure of this membrane receptor due to the change in cell membrane fluidity (Chachisvilis, Zhang, and Frangos 2006). After shear sensing, $G\beta/\gamma$ activates JNK, while $G\alpha_i$ activates ERK1/2 (S et al. 2003). In turn, ERK activation will lead to the increase in the intracellular calcium levels and the production of NO (Yan et al. 1999).

Another pathway activated by shear stress is the Akt pathway activated by PI3K, where PI3k is transiently activated after shear sensation by the receptors mentioned previously. A longer activation of Akt (6h) will

phosphorylate eNOS, thus allowing the production of NO that is stable for a longer period of time (Stefanie Dimmeler, Fleming, et al. 1999). This signaling pathway also changes the cells fate, as Akt is also involved in cell proliferation (Rössig et al. 2001) and protection against apoptotic stimuli (Conway et al. 2013).

For instance, different types of flow could differently induce miRNAs profiles that will either result in either physiological or pathophysiological functions (Marin et al. 2013). NOX activation, with its two major forms NOX2 and NOX4, is a major source of ROS production in ECs. Several studies have shown that many NOX components are regulated by miRNAs, where miR25 (Fu et al. 2010) and miR23-b (K.-C. Wang et al. 2010) have been validated in its NOX regulatory effect in ECs. So, after shear exposure, miRNAs fine tune ROS production through regulation of NOX subunits. On the other hand, high shear stress also increase the expression of miR-21, which will reduce the expression of pTEN, and increase phosphorylation of eNOS thus, increasing the production of NO (Weber et al. 2010).

Likewise, RhoA and Cdc42, members of the Rho family GTPases, are activated after SS exposure where integrins and extracellular matrix interactions are required for their activation (Tzima et al. 2001). In turn, RhoA and Cdc42 will activate JNK/AP-1, NF- κ B, and cFos that will regulate ET-1, MCP-1, inflammatory cytokines and adhesion molecules. (Johnson, Mather, and Wallace 2011). Besides, RhoA participates in the alignment of the ECs in the direction of flow during shear exposure (S. Li et al. 1999). But, under high shear stress, the cells are not only aligned in the direction of flow but are also polarized with a localization of the center of the microtubules upstream the nucleus. Numerous proteins of the peri-nuclear cytoskeleton including nesprin-3, activated by shear stress, regulate this localization (Morgan et al. 2011).

Finally, MAPKs also have a role of activating various transcription factors such as c-myc, AP1, or even NF κ B. In the inactive state, NF κ B is associated with I κ B, but the phosphorylation of I κ B by IKK (I κ B kinase) permits its degradation and the liberation of NF κ B, which will then be free to migrate to the nucleus. The strong shear stress will be able to transiently activate NF κ B by MAPKs but also by the VEGFR2-Akt pathway (Yingxiao Wang et al. 2004). However, KLF-2 and KLF-4 play an important role in the anti-inflammatory processes activated by the high shear stress (Hamik et al. 2007) and in the production of NO (SenBanerjee et al. 2004). The activation of KLF2 by the high shear stress makes it possible to maintain the quiescent character of the endothelial cells, in particular by increasing the expression of NOS protein (Dekker et al. 2002). In contrary, KLF-2 expression could be regulated also by MEK-5 and ERK-5, types of MAPKs. But also, it have been shown that shear stress KLF-2 expression would be the regulated by miR-92a (Essayagh et al. 2005) and by the cilia present on the surface of the endothelium (Figure 17) (Hierck et al. 2008).

where a tyrosine family kinase will transactivates VEGFR-2. Once VEGFR-2 is activated, it recruits PI3K that mediates the activation of Akt and eNOS (Jin Zheng-Gen et al. 2003).

Activating Rho and Rac family GTPases through the binding to integrins.

Rho GTPase signaling cascade is activated by shear stress through the activity of membrane integrins. Integrins $\alpha 5\beta 3$ increases its affinity after shear exposure to allow new integrins to bind to the ECM, where these connections are essential for the downstream signaling of Rho (Tzima et al. 2001). Rho acts on its effector protein p160ROCK that regulates stress fiber formation and cell alignment (S. Li et al. 1999).

PIEZO1 activation after shear mechanosensation.

When PIEZO-1 senses flow, a PIEZO-1 dependent calcium influx occurs in the cell. This increase in the intracellular calcium levels activates several pathways that influence endothelial cells alignment or shares common signaling pathways to alter endothelial cells morphology (Ranade et al. 2014).

In vitro, the impact of strong shear stress on the alignment of cells have a major effect on its morphology. Shear stress permits an endothelial cell to favor a specific hydrodynamic conformation. Endothelial cells alignment takes place in 3 phases (Galbraith, Skalak, and Chien 1998).

- First phase, this phase starts once the cells are subjected to flow (0-3 hours), where the cells lengthen through the formation of stress fibers and microfilaments. During this phase, intercellular junctions break inside each cell.
- Second phase, could be classified as an intermediate phase (3-6 hours), where the cells could move through the microtubules where the nucleus migrates to the upstream area of the cell.
- Third phase, after 6 to 12 hours, endothelial cells complete the alignment by aligning the intracellular stress fibers with each other and by restoring the intercellular junctions.

Once the stress fibers reappears, the cell significantly aligns in the direction of flow till it reaches its maximal alignment as it is exposed more to flow (Levesque and Nerem 1985). It should be noted that depending on the type of flow, endothelial cells are capable to align differently through the modulation in the activated pathway activity. For example, in the large vessels, which are exposed to high laminar flow, endothelial cells are well aligned. In contrast, at branching points, where the shear stress is low or oscillatory, these cells lack alignment (M et al. 1998).

6.2. Impact on cell fate

Different types of shear stress have different effects on ECs survival and apoptosis (Kadohama et al. 2007). However, Physiological shear stress has been defined as an apoptotic suppressor for the endothelial cells.

It has been reported that laminar shear stress suppresses the apoptosis through the activation of PI3K/Akt signaling pathway (Haga et al. 2003). Indeed, TNF α , ROS (reactive oxygen species) or oxLDL no longer able to induce apoptosis of endothelial cells once they are exposed to shear stress, this cell survival would be mediated by the inhibition of IL1 β , DAPK (death associated protein kinase), and caspase 3 (Freed and Greene 2010; Stefanie Dimmeler, Hermann, et al. 1999). The early exposure of ECs to shear stress would also block the activation of JNK by TNF α , inhibiting its pro-inflammatory effects. Under high shear stress, the production of NO is the major element mediating all the protective effects of shear stress against apoptosis (S. Dimmeler et al. 1998). Also, physiological shear stress also induce the expression of proteins inhibiting apoptosis (IAP-1 and -2). The activation of VEGFR2 by high shear stress contributes also to the protection against apoptosis (dela Paz et al. 2012).

6.3. Impact on cell migration

This step could be classified as the first stage in endothelial cells alignment process. After flow exposure, endothelial cells round up, loose their orientation, respreads and migrate within the direction of flow. This migration process is mediated by small Rho GTPases (Wojciak-Stothard 2011). During migration, the shape of the core could be greatly affected due to the mechanical stress generated by the cytoskeleton or emanating from the microenvironment. It is now well recognized that the cellular response to changes in mechanical properties of the environment is largely related to the remodeling of the cytoskeleton via the generated tension of the cell or the maturation of adhesions (Hoffman, Grashoff, and Schwartz 2011). In a confluent mat of cells, strong shear stress induce mobility (Tardy et al. 1997). After an endothelial injure, it has been shown in vitro and in vivo that recolonization by neighboring endothelial cells is very rapid under high shear stress (Sprague, Luo, and Palmaz 1997). Besides, endothelial membrane fluidity plays an important role in migration, which makes cholesterol membrane content as a determinant in this process.

6.4. Impact on cell proliferation

Proliferation is another of the mechanisms suppressed by laminar shear stress, whereas disturbed flow accelerates ECs proliferation and turnover process (Chiu and Chien 2011). Thus, physiological shear stress slows DNA replication, where the cells stop in the G0-G1 phase of the cell cycle mainly through the regulation of P53, which in turn will activate p21^{Cip1} that plays a role in the inhibition of cell proliferation through blocking cdk2 activation and pRb (Akimoto Shigeo et al. 2000). In addition, certain microRNAs contribute to this process, miR-19a increased by the strong shear stress represses the expression of cyclin-D1 stopping the cell cycle in G1 phase (Qin et al. 2010). Also, as shear stress activates both MAPK and Akt, this allows fine regulation of the cycle between the antimitotic effect of MAPK and the proliferative effect of Akt (D. Guo, Chien, and Shyy 2007). In vitro, the effect of shear stress appears to be dose-dependent: the greater the stress is, the less the cells proliferate (Levesque, Nerem, and Sprague 1990).

Finally, endogenous NO has not shown a role in the regulation of endothelial cells proliferation while the exogenous NO has a strong anti-proliferative effect (Gooch, Dangler, and Frangos 1997).

6.5. Impact on cell permeability

Endothelium plays a role of barrier for the transportation of solutes. Shear stress was shown to alter endothelium permeability mediated by transport pathways that are affected by shear stress. tight junctions, Adherens junctions, leaky junctions, and vesicles transportation are all affected by the shear stress under normal conditions (Tarbell 2010). Although, LDL is too large to pass through the breaks in the tight junctions, it is still able to pass through the leaky junctions under pathological conditions. In the vascular endothelium, the areas subjected to high shear stress are impermeable to macromolecules whereas the areas of low shear stress are acutely permeable to LDL (Stemerman M B et al. 1986). This permeability depends on the forces applied and is also reversible, depending on the time of exposure (Jo et al. 1991). In vitro studies have furthermore made it possible to demonstrate that the rearrangements of the endothelial cells to align themselves in the direction of flow lead to an increase in permeability. After the cells reach their maximal alignment, cell permeability is returned to its normal state (Seebach et al. 2007). At the intracellular level, the short-term effect is mediated by a decrease in the VE-cadherin or occludins, as endothelial cells migrate and align in the flow direction (Pang, Antonetti, and Tarbell 2005). Chang et al. demonstrated that this short-term effect under high shear stress could be NO-dependent, and that cAMP would counteract this increase (Chang Yong S. et al. 2000). On the other hand, the decrease in long-term permeability would pass through the activation of the PI3K-NO-cGMP pathway (Warboys et al. 2010). Therefore, NO could play a dual role on endothelial permeability, increasing short-term permeability, by acting on cAMP and by decreasing long-term permeability by mobilization of cGMP.

As a conclusion, endothelial cells then play their role as a barrier, mediator of vasomotricity and modulator of hemostasis. Although, all the above events take place under physiological conditions where the flow is laminar, there are areas of the vascular tree naturally subject to lower disturbed or oscillatory shear stress due to the shape of the vessels which can modify local flows. Thus, mechanotransduction is activated in a different manner promoting the appearance of various vascular pathologies including atherosclerosis.

7. Endothelial Dysfunction: case of Atherosclerosis

It is necessary to mention that in our study we have used atherosclerotic models as models presenting vascular pathology caused by endothelial dysfunctions due to a variation in the endothelium mechanotransduction.

7.1. Definition of Atherosclerosis

Atherosclerosis is a chronic pathophysiological disease that could occur in slow progression early in the childhood and evolves asymptotically throughout aging, with atherosclerotic plaques accumulating in the arteries. Subsequently, with age, it is clinically reflected by the occurrence of acute ischemic attacks (heart, brain, critical limb ischemia). These lipid plaques can rupture in late stages of this pathology, leading to thrombosis. This leads to a change in the tunica intima of large and medium sized arteries, where it accumulates lipids, complex carbohydrates, blood products, fibrous tissues and calcareous deposits that all leads to a change in the tunica media. Depending on the location of the thrombus, this can cause a variety of complications, including events like stroke or myocardial infarction.

This disease represents the first cause of world mortality. In 2015, atherosclerotic deaths were reported to be 31% of global deaths worldwide. Within the United States and Europe, this pathology accounts for 33 to 40% of all-cause mortality. According to the WHO, an estimated 17.3 million deaths are attributable to cardiovascular diseases, or 30% of the total global mortality. Ischemic heart disease and stroke have respectively caused 7.4 and 5.1 million deaths in 1998. In France, it is responsible each year for 120,000 myocardial infarctions, 130,000 strokes and 60,000 hospitalizations for revascularization of the lower limbs with around 10,000 major amputations. Due to lifestyle changes and the overall aging of the population, atherosclerosis, or rather its pathologic consequences, are in full expansion: by 2030, 23 million people will die of cardiovascular disease. It is therefore one of the major issues in research and in current medicine. The increase in these numbers could be due to three factors: The first factor taken into account in atherosclerotic pathophysiology is the population slump, with a correlation between age and heredity in a population. The second factor is the explosion in the prevalence of obesity and type 2 diabetes. The third factor is the new lifestyle adopted by the new generations, characterized by an increase in sedentary lifestyles, poor diet (fast food) and smoking, which promotes excess weight at an early age (Bonow Robert O. et al. 2002).

Atherosclerotic lesions initially develop at specific sites in the arteries, which are susceptible to atherosclerosis and exhibiting intimal thickening (Nakashima et al. 2002). These privileged sites are found mainly at the bifurcations of the arteries and on the internal face of the vascular curvatures. At these sites, the blood flow is disturbed and leaves the laminar state with the consequence of a decrease and an irregularity of the applied shear stress on endothelial cells, participating in endothelial dysfunction recognized as an early event of atherogenesis (Malek, Alper, and Izumo 1999).

7.2. Atherosclerosis risk factors

Different elements and variables participate in the appearance and development of atherosclerosis. These factors are diverse in terms of origin and of their rate of aiding in atherogenesis. They could be categorized as follows:

7.2.1. Lifestyle factors:

Lifestyle plays an important role in the progression of atherosclerosis. During daily life, people are practicing different activities such as:

Smoking:

Tobacco contains nicotine that promotes sudden narrowing of the arteries. It stimulates adrenergic receptor α_1 , which favors arterial vasoconstriction. The toxins present in a cigarette increases inflammation of vascular cells, oxidative stress and thrombogenesis. Besides, smoking is also associated with an augmentation in the levels of expression of ICAM-1 and TNF- α in ECs, which leads to an endothelial dysfunction (Blann, Steele, and McCollum 1997).

Sedentary lifestyle:

Lack of physical activity decreases the resistance of the arteries. ApoE^{-/-} mice subjected to physical activity were less likely to develop atherosclerosis, which is due to the decrease in the production of ROS (Okabe et al. 2007). Another studies have demonstrated that the physical activities, in mice model, can increase the number of endothelial progenitor cells (EPCs) that are essential for EC regeneration and angiogenesis (Laufs et al. 2004).

Diet:

A diet rich in fats and sugars will promote the development of pathologies associated with atherosclerosis such as obesity or diabetes.

Stress:

Under stress conditions, the body secretes more adrenaline. High adrenaline levels causes the arteries to spasm. When the stress is repeated too often, it can lead to hypertension and repeated arterial spasms that ends in a perturbation in the vascular system and favors atherosclerosis.

2.3.3. Genetic Factors

Atherosclerosis can also have a genetic component. Even if no specific mutation or gene was identified, the influences being multiple.

Gender influences susceptibility to atherosclerosis, where men are more at risk than women do. This difference diminishes after menopause.

Familial hypercholesterolemia is the most common genetic disease in atherosclerosis, which is caused by a defect in chromosome 19. Patient body with this disease have no capability to remove LDL cholesterol from the blood. A study has also shown that genetic variation or a mutation in the eNOS gene (and therefore probably variations in the bioavailability of NO) is a major cause of this disorder (Casas et al. 2004). Besides, about 30% of coronary heart disease patients have lost their fathers with the same disease.

2.3.4. Metabolic pathologies:

Certain pathologies contribute largely to the development of atherosclerosis:

Hypertension

It is due to an increase in total peripheral resistance and leads to vascular remodeling that makes the arteries more rigid. Interestingly, just as hypertension worsens atherosclerosis, atherosclerosis complicates and worsens hypertension. Thus, hypertension and atherosclerosis are two vascular pathologies that both can trigger each other.

Diabetes:

Epidemiological data establish with certainty the correlation between type 1 or 2 diabetes and the occurrence of coronary heart disease (Pyörälä, Laakso, and Uusitupa 1987). The increase of coronary diseases in diabetic patients was not determined precisely, but various actors have been implicated. Excess insulin secretion seems to promote the proliferation of SMCs by stimulating the mitogenic action of growth factors like PGDFs and participates in the increase in synthesis matrix proteins of the vascular wall. Hyperglycemia can induce the formation of advanced glycation products and a modification in the oxidative stress and ROS production. These components are likely to generate an inflammatory state conducive to the initiation of atherosclerosis. In particular, advanced glycation products exert a role in the activation of ECs by increasing the expression of adhesion molecules to their surface and by increasing the expression of adhesion molecules to their surface, and by stimulating the pathway of NF- κ B (Ramasamy et al. 2005).

Hypercholesterolemia:

Epidemiological studies showed that high levels of LDL-cholesterol and triglyceride are risk factors of atherosclerosis (Waters David et al. 1995). In addition, a relation was reported between cholesterol rich diet (animal lipids) and atherosclerosis. Circulating LDL can infiltrate the subendothelium and accumulate there

for several reasons identified. Thus, the lipoproteins will be trapped in the intima where it undergoes oxidative modifications and become oxidized LDL (ox-LDL). In addition to their effects on the formation of foam cells, oxidized LDL also acts on endothelial cells and smooth muscle cells of the vascular wall by inducing the expression of pro-inflammatory genes (Boullier et al. 2001).

Obesity:

If excess weight does not seem to play a direct role in the onset of atherosclerosis, it is very often associated with diabetes, increased cholesterol and high blood pressure, which are all mentioned above as risk factors for atherosclerosis. The combination of at least one of these factors with another, leads to the metabolic syndrome. However, the presence of excess fatty tissue is positively correlated with chronic inflammatory conditions. The adipose tissues, which are highly present in obese people, produces inflammatory molecules such as leptin, adiponectin, IL-6, and TNF- α (Weisberg et al. 2003).

7.3. Major steps of Atherosclerosis

Lipoproteins are key players implicated in atherosclerosis. Plasma lipoproteins are particles containing proteins, cholesterol and other lipids that allow their distribution through the body until their metabolization or degradation. The plasma lipoproteins are traditionally separated between high-density lipoproteins HDL (also known as “good cholesterol”) and the low-density lipoproteins LDL (the infamous “bad cholesterol”).

Under laminar flow, two main roles of endothelial cells to respond to flow: the first is the production of NO, and the second is the expression of the antioxidant superoxide dismutase. These two functions have an anti-atherosclerotic role in the vessels (more detailed under the next title).

The onset of atherosclerosis starts when LDL transigrate through the endothelial monolayer into the intimal space (the space between the intima and the media). Those LDL are trapped in the intimal space and undergo oxidative or enzymatic modifications. Altered LDL causes an inflammatory reaction that activates the endothelial cells. The activated endothelial cells express vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and selectin adhesion molecules (CD62), which participate in the recruitment of the monocytes from the circulation into the intimal space and their differentiation into macrophages (Schrijvers et al. 2007). Recruited macrophages express at their plasma membrane, among others, a panel of receptors called the scavenger receptors (SRB1, CD36, CD68...) that are able to recognize danger-associated molecular patterns. So, they recognize modified LDL and allow its uptake by the macrophages (Silverstein 2009). The macrophages accumulate the LDL in their cytoplasm, transforming them into foam cells. Small conglomerates of foam cells are considered as the first stage of atherosclerosis as described first by Stary in 1994 (Stary H C et al. 1994).

Foam cells secrete various inflammatory cytokines that further activate endothelium permitting increased recruitment of monocytes and thus leading to the formation of additional foam cells, which form the fatty streaks, visible as a yellow linear protrusion in the intima (stage II) (Swirski et al. 2006).

At this stage, the inflammation triggered by the foam cells also modifies the phenotype of the smooth muscle cells (SMC) in the intima. These SMCs are quiescent and contractile under normal physiological condition, but after activation by PDGF (Platelet Derived Growth Factor) released by the foam cells they start to migrate into the intimal space, where they proliferate and secrete cytokines (their functions changes from contractile profile to secretory profile). The fatty streaks are mostly localized at the arterial bifurcations, where the shear stress is lower and oscillatory, and can in turn become pre-atherosclerotic lesions, in which small pools of extracellular lipids accumulate in the neointimal space (stage III) (Mosse et al. 1985).

However, the accumulation of monocytes derived macrophages is irreversible as they cannot return to circulation, thus they die directly in the plaque either by necrosis or apoptosis (both have been observed within the plaque) and ultimately liberate their lipid content in the intimal space. This leads to the formation of the necrotic core, an acellular accumulation of crystalized cholesterol and dead cell debris, surrounded by numerous living foam cells (stage IV) (A. P. Burke et al. 2001).

Dedifferentiated smooth muscle cells secrete an important quantity of fibrous proteins that form the fibrous cap, a layer of connective tissue that separate the necrotic core from the intima. The result is a fibro-lipid plaque (stage V). At this stage the intima is still present but the initial connective tissue on which it was lying is already degraded. Stage V plaques can have disruptions at their surface, such as hematoma or hemorrhage; in that case they are considered complicated lesions (stage VI) (Figure 18)

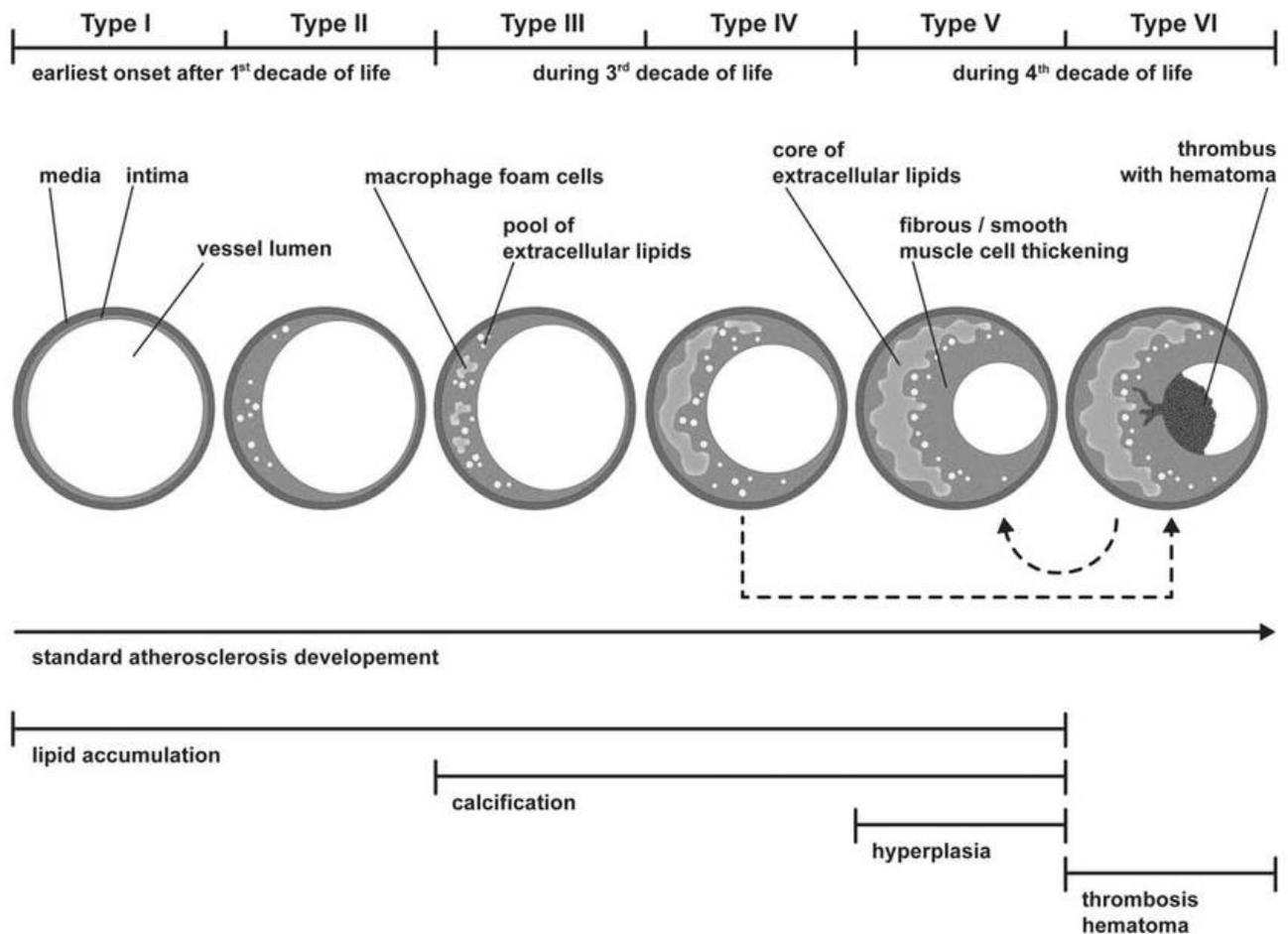


Figure 18 Stages in the development of atherosclerosis with age (Limbach et al. 2012)

Finally, some of the plaques may calcify (stage VII) which impairs the contractile function of the vessels. Finally, there are plaques, which are highly fibrotic with very low lipid content and are classified as type VIII (Stary H C et al. 1994).

8. Shear stress and atherosclerosis progression

Mechanical irritation of the vascular wall is one of the main causes of atherosclerosis. It is due to the mechanical aggression of the vascular wall due to stress conditions imposed by the pulsation of the blood flow. It was not until the twentieth century that there were two postulates on mechanical stress in order to explain the distribution of lesions within the vascular tree: One assumed that strong shear stress are generated by a laminar flow, while the other appreciated that oscillatory or turbulent flow generated weak shear stress.

However, studies have demonstrated that atherosclerotic plaques are favored in the zones subjected to low or perturbed flow, whereas in the zones of laminar flow there was a little to no atherosclerotic plaque

formed. This is because, under normal conditions, the surface of the vascular endothelium responds normally to the shear stress avoiding endothelial dysfunction. Constant laminar shear stress due to blood flow maintain the endothelial cells in an anti-inflammatory, anti-oxidant and antithrombotic phenotype. In contrast, the inflammatory activation of the endothelium occurs at the level of the branching sites of arteries, and in particular, at the level of the arterial bifurcations, the curvatures and the emission point of the branches where endothelial surface is subjected to low or oscillatory shear stress (Figure 19) (Gimbrone et al. 2000).

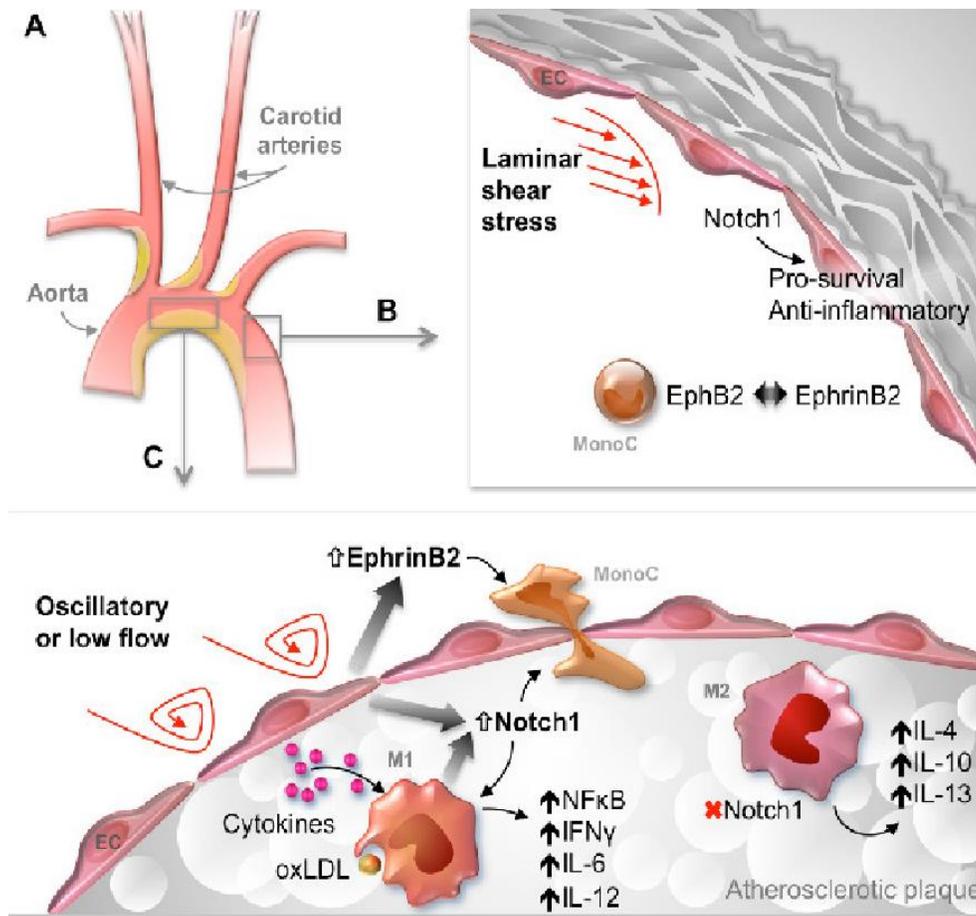


Figure 19 Signaling pathways activated by endothelial cells in different zones subjected to plaque formation (A) due to LSS (B) and OSS or low shear stress (C) (Lehoux and Jones 2016).

Chapter 3:
Mitochondria
&
Mitochondrial
Dynamics

1. The mitochondrion

1.1. Mitochondrial structure

The mitochondria, descendants of α -proteobacteria, are semi-autonomous membrane-bound organelles present within the cytoplasm of eukaryotic cells. In human cells, the number of mitochondria may differ from one cell to another where hundreds to thousands of mitochondria could be found, depending on the cell type. The number of mitochondria is proportional to the energy required for the cell, the more the organ needs energy, the more mitochondria it will have, where muscle, heart, and liver cells are the richest in mitochondria.

The size and shape of mitochondria varies depending on the metabolic condition and cell type. But, in average, they measure 0.5 to 1 μm in diameter. Because of this double-membrane organization, four distinct parts in the mitochondrion could be distinguished: the outer mitochondrial membrane OMM, intermembrane space IMS, inner mitochondrial membrane IMM, and the mitochondrial matrix MM (Low 1956).

The two mitochondrial membranes have very different properties and composition, where the outer membrane (5-7 nm thickness) is composed of 40% lipids (mainly unsaturated phospholipids and some cholesterol) and of 60% proteins, it is highly permeable to ions and small molecules as it is rich in porin, called VDAC (voltage-dependent anion channel). However, the IMM is composed of 75% proteins (mostly hydrophobic) and 25% lipids, with a low cholesterol level less than 3% and a high cardiolipin concentration 20% of the total lipids of IMM. Besides, IMM is convoluted into many internal invaginations called cristae where the mitochondrial respiratory chains complexes are located. Most of the proteins constituting IMM are transporters of the ETC, enzymes for the synthesis of steroids, permeases and translocases.

Mitochondria have their own genome (mtDNA) as well as a replication, transcription, and translation system which is independent of the nuclear machinery. The mitochondrial matrix contains many enzymes which are involved in the energy metabolism like Krebs cycle, β -oxidation of fatty acids, urea cycle and ketogenesis enzymes. Also, mitochondrial matrix contains multiple copies of mtDNA as well as the components necessary for the replication and translation machinery including: rRNA, tRNA, ADP, ATP, mito-ribosomes and all the enzymes involved in mtDNA repairing.

1.2. Main functions of the mitochondria

Mitochondria perform many vital functions in the cell with a central role in cellular bioenergetics and signaling transduction. In eukaryotic cells, the mitochondrial prominent role is to produce energy, as it is

known by the energy factory or the powerhouse of the cell. Although several functions could be attributed to the mitochondria (Dorn II 2015), it participates in main physiological processes such as:

- Oxidative phosphorylation (OXPHOS): Main process involved in the production of 90% of ATP in the cell.
- Apoptosis or necrosis: cell death.
- Oxidative stress: generation of reactive oxygen species ROS.
- β -oxidation of fatty acids
- Krebs cycle
- Ion homeostasis
- Calcium signaling
- Thermogenesis

However, in this chapter, only oxidative phosphorylation, oxidative stress and cell death will be detailed.

1.2.1. Oxidative phosphorylation

Most of the energy produced by the cell is produced through oxidative phosphorylation, which is also known as the cellular respiration process. This process occurs in the cristae of the IMM, where the ETC is located. ETC consists of a series of complex proteins that transport electrons from electron donors to electron acceptors in a series of redox reactions. A proton gradient is generated as a result of the electron passage through these complexes, where this gradient is used for the generation of ATP by chemiosmosis. This process is called oxidative phosphorylation.

Pyruvate and Acyl co-A, produced from carbohydrates and fatty acids, are imported into the mitochondrial matrix to be transformed to Acetyl-coA. These two metabolites are catabolized in the matrix through the decarboxylation of pyruvate by pyruvate dehydrogenase and β -oxidation of Acyl co-A. After the integration of Acetyl co-A into the Krebs cycle, it produces reduced cofactors NADH, H^+ , and FADH₂. These latter are considered as reduced electron carriers.

The NADH, H^+ , and FADH₂ released from Krebs cycle are then oxidized at the level of complex I and II of the ETC, known as NADH dehydrogenase and succinate dehydrogenase respectively. NAD⁺ and FAD⁺, which are produced from the action of the first two complexes, are then reused in other steps of cellular respiration. Afterward, the electrons generated are then passed down along the mitochondrial respiratory chain, where they move from high to low energy levels until they reach complex IV, which will then reduce liberated oxygen into water molecules. All of the electron passages through the complexes I, III and IV, are coupled to the translocation of H^+ ions from the matrix to the IMS. As a result of this coupling, a proton

gradient in the IMS generates a proto-motive force across the IMM and H^+ starts to pass through the complex V (ATP synthase), a molecular rotor that triggers the production of ATP from ADP and inorganic phosphate (Mitchell 1961).

In addition to the production of ATP, oxidative phosphorylation is also a main source of ROS in the cell; these free radicals are in the form O_2^- , H_2O_2 and HO^\cdot . The ROS correspond to all the molecules deriving from the activation of molecular oxygen. These species are unstable, neutral, or charged as they possess a single electron generated due to the fact that some electrons evade the mitochondrial respiratory chain and react with molecular oxygen. Although ROS play an essential role as signaling molecules in response to several stress on the cells, they can be harmful and toxic to the cell when they are produced in quantities that exceed the potentials of antioxidant systems in the cells. These systems are characterized mainly by catalase and superoxide dismutase 1,2,3 (SOD1,2,3) that sense and act as endogenous defense mechanisms to detoxify ROS once liberated (Berg, Tymoczko, and Stryer 2002). All of these processes taking place in the mitochondrial respiratory chain are shown below in Figure 20.

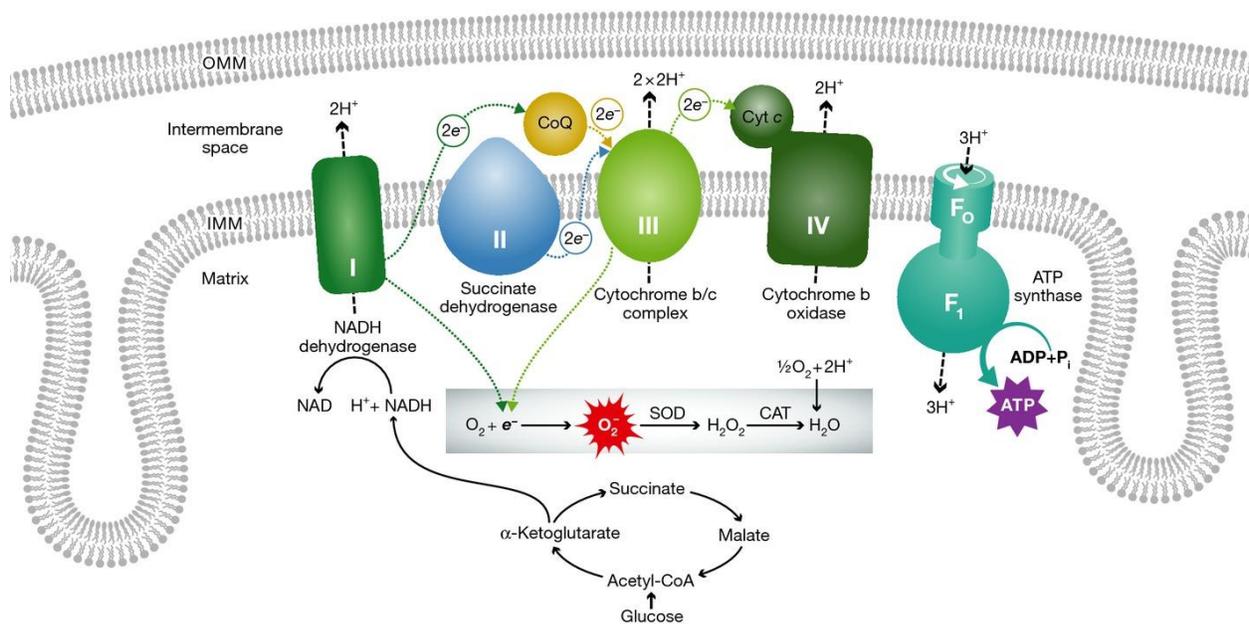


Figure 20 The mitochondrial electron transport chain. The four complexes of the ETC are anchored to the IMM, complex I and II oxidize the reduced factors produced from the TCA cycle to generate electrons e^- and protons H^+ . Then, Electrons are transferred and passed between complexes I and IV, where they induce the transport of H^+ from the matrix to the IMS. Proton gradient in the IMS generates a protomotive force that activates complex V of the ETC that will synthesize ATP from ADP through its rotatory activity. However, some electrons escape the ETC and use O_2 molecules to generate O_2^- , H_2O_2 , and HO^\cdot (Dorn II 2015).

1.2.2. Oxidative Stress

An imbalance in the free radicals formation and antioxidants of the body causes oxidative stress. The main actors in the oxidative stress process are the antioxidant enzymes SODs and catalases. Oxidative stress occurs when the formation of oxidant molecules is favored on the activity of antioxidant enzymes. This situation may result from a deficiency or failure in antioxidant systems, disturbance in the production, distribution, or increased abundance of free radicals (Pincemail et al. 2002). A free radical is characterized by its instability and high reactivity with a very short lifespan. These latter tend to fill its orbital by capturing an electron by the oxidation of other compounds like nucleic acids, lipids, proteins, and carbohydrates (Goudable and Favier 1997).

As mentioned above, ROS are not only responsible for cell cytotoxicity, but also they act in different signaling pathways in the cell. The levels of ROS are controlled by SOD to limit their cytotoxicity and regulate their signaling activities. SOD is a detoxifying metalloprotein and antioxidant defense in all the living cells exposed to oxygen (except for lactobacilli). SOD acts by catalyzing the dismutation of superoxide free radicals into oxygen and hydrogen peroxide. Three major families of SODs could be distinguished, which are encoded by different genes with different localizations inside the cell. Depending on the encoded SOD and the metal cofactor bound, we can distinguish (Ighodaro and Akinloye 2018):

- SOD1, also known as Cu/Zn type where SOD binds to Copper and Zinc as cofactors, and localized mainly in the cytosol of the cells, it is the most stable form of SOD as it is bound to highly stable cofactors.
- SOD2 or MnSOD, also could bind to iron (Fe) and manganese (Mn), this type is only localized in the mitochondria.
- SOD3 or Cu-ZnSOD, this type is extracellularly complexed with copper and zinc cations (in humans).

On the contrary, the H_2O_2 liberated from SOD reaction is toxic and can damage the cell. That's why hydrogen peroxide is then directly degraded by other enzymes like catalases. This anti-oxidant enzyme is 240 KDa tetrameric protein that is localized in peroxisomes in mammals. It uses the H_2O_2 liberated during fatty acid oxidation in the peroxisomes. It uses either iron or manganese as a cofactor, and accomplish its function by the aid of glutathione peroxidases (GPXs) and peroxiredoxins (PRXs) (J. Zhang et al. 2016).

For instance, O_2^- is one form of ROS that results from different endogenous processes in the cell. This radical is dismutated by SOD into H_2O_2 that reacts with iron Fe^{2+} to produce OH^* . But, O_2^- can also react with other free radicals like NO^* and liberate $ONOO^-$, which is the origin of some highly oxidizing intermediates shown in Figure 21 (Ying Wang et al. 2018).

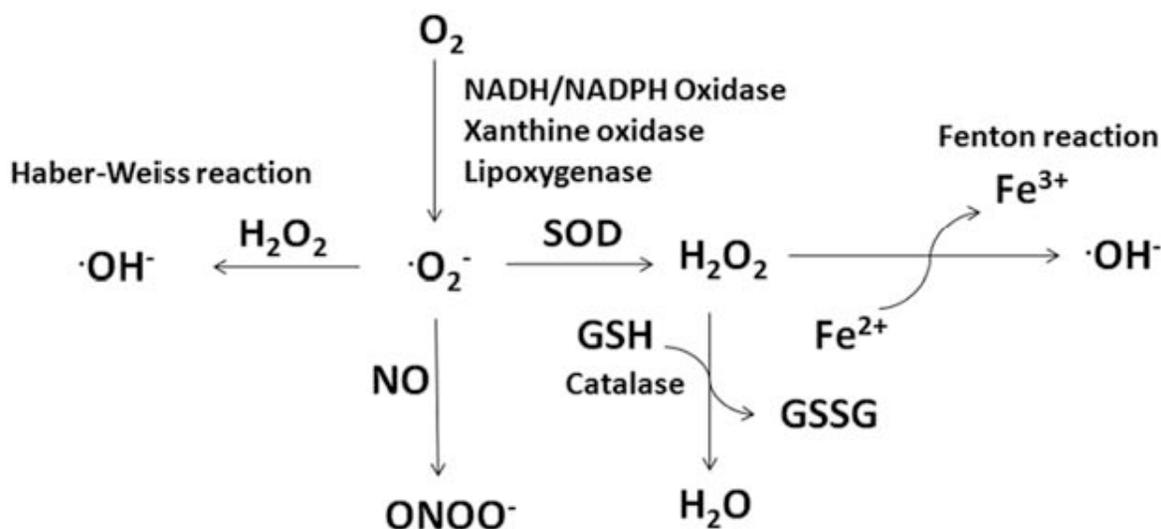


Figure 21 Schematic representation for the oxidative stress inside the cell where Superoxide Dismutase (SOD) and catalase are the main actors in detoxifying the cell toxic radicals (Boda and Basu 2017)

1.2.3. Cell death

One of the main functions of the mitochondria is the regulation of cell death. Although the mitochondria are the powerhouse of the cell and its production of energy is essential for cell viability, different signals activated by endogenous and exogenous factors lead to the participation of the mitochondria in the integration and propagation of death signals. Two main processes by which cells could die, either by cell apoptosis or cell necrosis. In vivo, the two processes are difficult to distinguish as they occur simultaneously in the tissues within the same pathophysiological context (Goldenthal 2016).

Cells apoptosis is the physiological mechanism by which the cell self-destroys itself in order to serve in a variety of biological events. It is an important programmed cell death for maintaining developmental sculpturing, tissue homeostasis, compensate mitosis, and removal of damaged cells. That is, cells that are senescent, dangerous and no longer needed, are destroyed by this highly regulated mechanism. A dis-regulation in this mechanism can contribute to various diseases such as ischemia, neurodegeneration, autoimmunity diseases, tumor regression, and viral infections (Saikumar et al. 1999). Two cellular pathways can trigger apoptosis, the intrinsic and the extrinsic pathway where the in the first pathway the mitochondrion has a central role through the pro- and anti-apoptotic Bcl-2 (B-cell lymphoma 2) family members, and the second consists of the TNF family receptors like TNF receptor, CD95/Fas and TRAIL death receptors (Ferri and Kroemer 2001).

An apoptotic signal induced by cellular stress leads to the permeabilization of the OMM by BAX (Bcl-2 associated protein X) and BAK (Bcl-2 antagonist). BAX and BAK associate to form pores on the OMM,

this will allow the release of cytochrome c from the IMS to the cytosol. Apoptosis intrinsic activation is executed by cysteine proteases that cleave their substrates at aspartic acid residues. This process starts with the formation of the cytosolic apoptosome after the cleavage of procaspases (inactive precursors of caspase) to activate them. The apoptosome is composed of Apaf-1 (Apoptosis Protein Activating Factor) and cytochrome c released from the mitochondria and binds to procaspase 9. After that, the apoptosome/procaspase activates caspase 9, which in turn activates other effector caspases like caspase 3, 6, and 7 that triggers the cell death process by degrading cellular substrates (Figure 22A) (Green and Evan 2002).

On the other hand, necrosis is an accidental cell death that usually takes place under pathological conditions. General cell swelling and in particular mitochondrial swelling characterizes this process. The swelling of the mitochondria leads to the rupture of the mitochondrial and cell membranes, and as a consequence a severe impairment in the energy production as well as inflammation. The change in the membranes permeability alter Bcl-2 functioning and thus, the mPTP opening will be no more controlled and inhibited by Bcl-2. The opening of mPTP is more facilitated after its binding to BAX, which will facilitate the passage of protons through the IMM that will result in a loss in the mitochondrial membrane potential (Ψ_m) and in the ATP production due to the loss in proton gradient used by ATP synthase during OXPHOS. As a result, the inner membrane swallows (water and solutes enter to the mitochondria) and leads to the rupture of the OMM that will result in the release of the cytotoxic proteins from the mitochondria into the cytosol (Figure 22B) (Kubli Dieter A. and Gustafsson Åsa B. 2012).

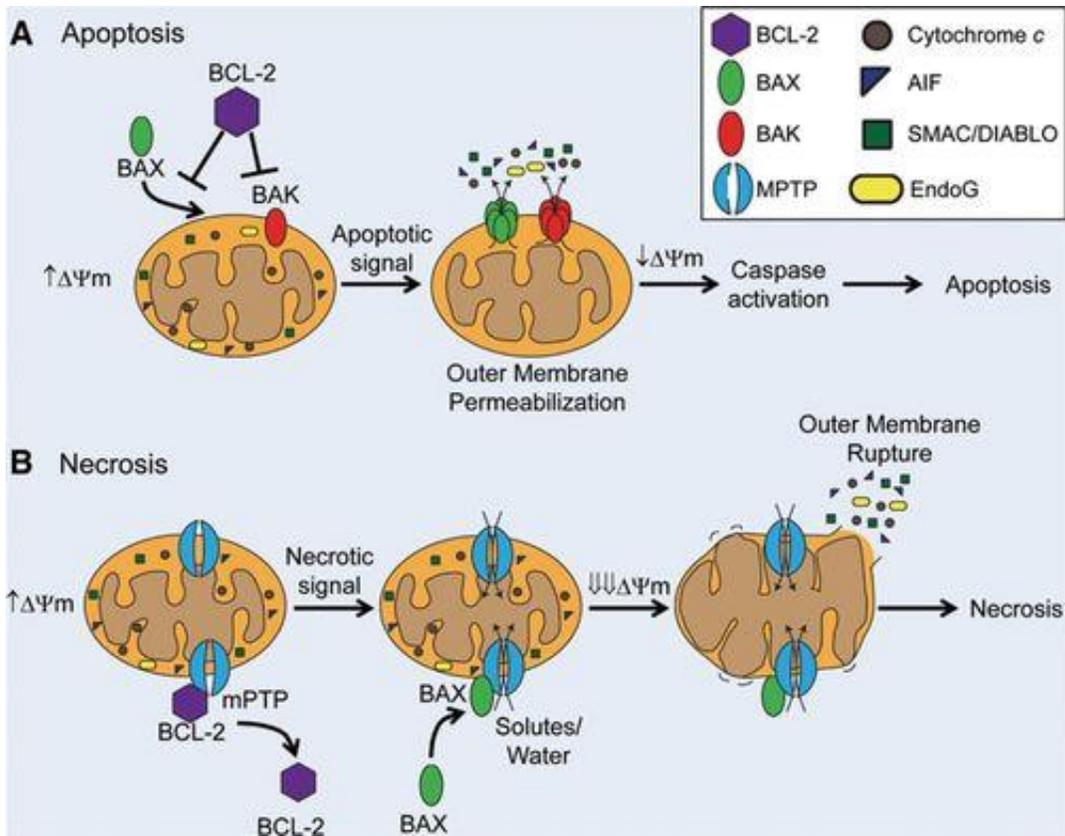


Figure 22 The implication of the mitochondrial signaling in the cell fate. (A) Intrinsic pathway activated by the mitochondria that leads to cell apoptosis. (B) Necrotic signaling in the cells that leads to inflammation and cell cytotoxicity (Kubli Dieter A. and Gustafsson Åsa B. 2012).

2. Mitochondrial Dynamics

2.1. Generalities

The mitochondrion was first considered as a static organelle for a long period of time. However, under the light microscope, these organelles appear in the form of rods, grains, or filaments of variable lengths. This was dependent on the cell type, where the same cells when visualized on video microscopy, show that they are constantly moving and along the cytoskeleton. The mitochondria are highly dynamic organelles undergoing fission and fusion in a highly regulated manner. Thus, they can divide, combine, and move along the cytoskeleton in order to meet cell metabolic needs, where the morphology of the mitochondria is linked to its functions, including the production of ATP through OXPHOS, apoptosis, and necrosis regulation, oxidative stress regulation.

The role of mitochondrial dynamics is to control the morphology, quantity, and quality of the mitochondria. Besides, mitochondrial dynamics intervenes with mitochondrial biogenesis, elimination, and distribution of the mitochondria within the cellular environment (McCarron et al. 2013).

This process is highly regulated to support cell homeostasis for maintaining the proper functioning of the complex organelles (Figure 23). Under stressful conditions like nutrient excess or disturbed shear stress, the mitochondrial dynamics balance shifts more toward mitochondrial fission that enhances cell death and impairs the functioning of the mitochondrial respiratory chain complexes. On the other hand, mild stress like mild starvation can favor mitochondrial fusion that was associated with an enhancement in the activity of the mitochondrial respiratory chain complexes (Wai and Langer 2016). The dysregulation in the scale between fusion and fission can lead to several pathologies that could be severe depending on the cell type and state (Wai and Langer 2016). These pathologies, including neurodegenerative diseases, cardiac hypertrophy, and other cardiovascular pathologies, could be due to an alteration in the balance in mitochondrial dynamic proteins that leads to the loss of the maintenance in the integrity of mtDNA, decrease in cell metabolism, and dysregulation in the mitochondrial quantity and quality (I. Kim, Rodriguez-Enriquez, and Lemasters 2007). During mitophagy, a selective mitochondrial autophagy process, an abnormal fusion to fission proteins can lead to mitochondrial dysfunction (Kunkel, Chaturvedi, and Tyagi 2015). However, most often, mitochondrial fusion is associated with an increase in ATP production while the inhibition of fusion is associated with a decrease in the production of ATP (Liesa and Shirihai 2013).

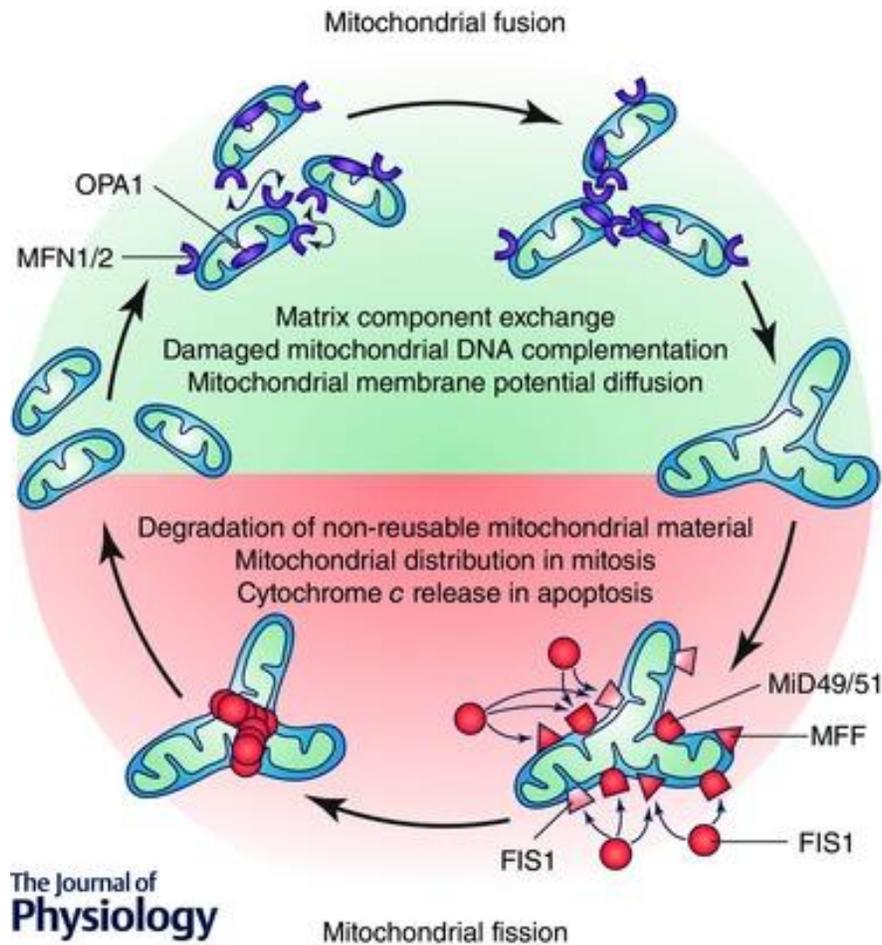


Figure 23 The shifting in the balance in mitochondrial dynamic process depends on the cellular needs and on the survival conditions of the cell (Vásquez-Trincado et al. 2016)

Mitochondrial dynamics proteins are not only specialized in the process of fusion and fission; however they interact with other proteins either to accomplish their functions or to participate in other cellular processes. For instance, both mitochondrial fusion and fission proteins are implicated in mitophagy, a selective mitochondrial autophagy. In contrast, mitochondrial fusion proteins are implicated in other processes like cell apoptosis, endoplasmic reticulum/mitochondrial tethering, and ensuring the alignment of the respiratory chain complexes (Figure 24) (Ong et al. 2017).

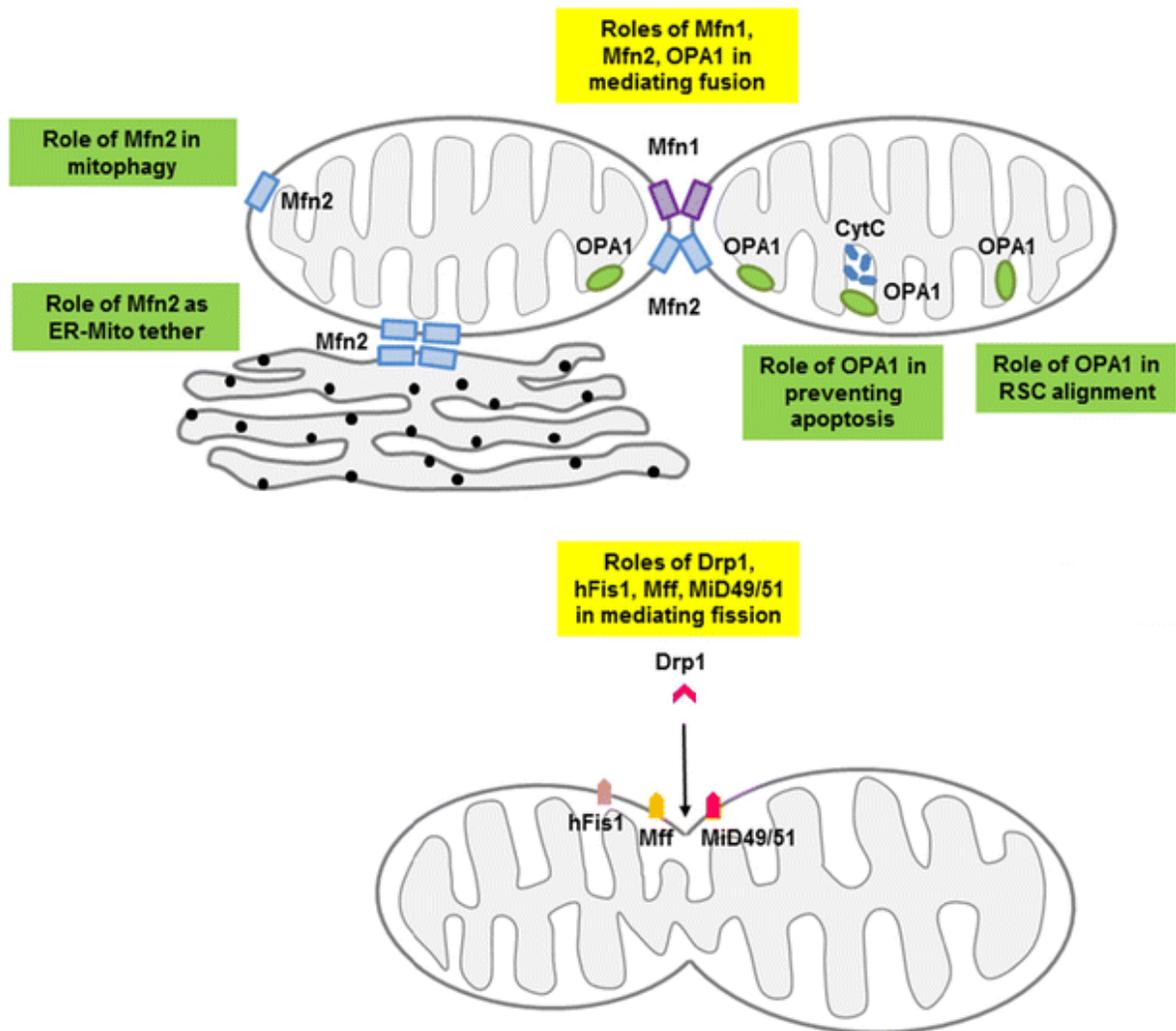


Figure 24 Implication of different mitochondrial dynamics proteins in various cellular processes. These proteins include mitochondrial fusion proteins (Mfn1/2, OPA1) and mitochondrial fission proteins (hFis1, DRP1, MFF, MiD49/51) (Ong et al. 2017).

2.2. Mitochondrial Fusion

Mitochondrial fusion is the process of combining two mitochondria to form one mitochondrion. It is an essential process for maintaining a homogenous mitochondrial population. It protects the organelles from degradation during starvation or cell apoptosis. This process occurs in two stages. First stage is the fusion of the outer membranes by the mitofusin proteins (MFN1 and MFN2) followed by a second stage of a fusion of the inner membranes under the control of optic atrophy protein 1 (OPA1) (Malka, Lombès, and Rojo 2006). Both MFN2 and OPA1 was shown to be essential in mitochondrial fusion where a frameshift and missense mutation results in higher mitochondrial fragmentation (Delettre et al. 2000; H. Chen et al. 2003).

2.2.1. Mitochondrial fusion proteins

Mitofusins MFN1 and MFN2

Mfn1 and Mfn2 are mitochondrial proteins that are encoded by nuclear genes. They are 85 KDa GTPases that belong to the dynamin superfamily and share 80% homology as well as similar structural patterns (Santel 2003). Besides, the two mitofusins share certain functional redundancy in mice (H. Chen et al. 2003). They are outer mitochondrial membrane proteins consisting of a GTPase domains (G1 to G5), two heptad repeated domains (HR1 and HR2), and a transmembrane domain (TM) (Figure 25) (Schrepfer and Scorrano 2016). As shown in Figure 25, mitofusins span the OMM twice through their transmembrane domains TM where each of the two mitochondria keeps the HR1 and GTPase domains exposed in the cytosol.

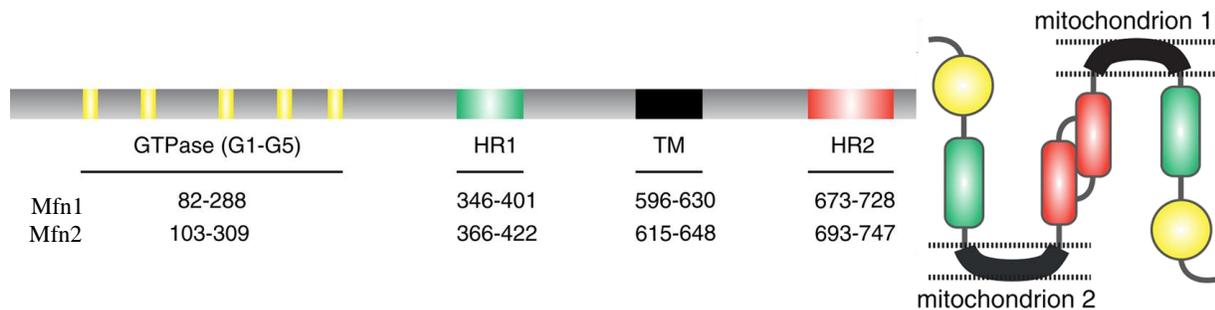


Figure 25 Structure of the mitofusins, where Mfn1 and Mfn2 are organized in the same sequence in structure. As shown in red, HR2 permits the tethering of two adjacent mitochondria through an antiparallel-coiled coil structure. These domain interactions could be either homotypic (Mfn1-Mfn1 and Mfn2-Mfn2) or heterotypic (Mfn1-Mfn2) (Chandhok, Lazarou, and Neumann 2018).

Chen et al., have shown in 2003 that the Mfn1 or Mfn2 knockout mice are alive whereas deletion of one of the two mitofusins results in embryonic lethality in mice (H. Chen et al. 2003). Also, as a result of Mfn2 deletion, a disturbance in placental development occurs. Whereas, when Mfn2 expression was suppressed after placentation, animals die after few days from birth due to brain malformations (H. Chen, McCaffery, and Chan 2007). In humans, Charcot-Marie-Tooth type 2A, a peripheral neuropathy characterized by axonal degeneration and distal muscle atrophy is caused after pathogenic mutation of Mfn2 and not Mfn1 (Burté et al. 2015). Indeed, Mfn1 compensates for the Mfn2 deficiency in all the tissues except for the peripheral nerves, where Mfn1 expression is low (Amiott et al. 2008).

Although Mfn1 and Mfn2 are regulated by post-translational modifications including phosphorylation and acetylation (Adaniya et al. 2019), its proteosomal degradation is regulated by ubiquitination and phosphorylation. This will serve in the mitochondrial quality control through the mitophagy process. The

ubiquitilation of Mfn2 via PINK1/Parkin pathway would enhance mitochondrial fission and recruit p62, the key player in mitophagy. Mitophagy is an important process that allows the elimination of altered mitochondria and thus it prevents the fusion of healthy mitochondria with non-healthy ones (Gegg et al. 2010).

Optic Atrophy factor 1 (OPA1)

It is a GTPase dynamin with about 100 KDa of molecular weight and resembles mitofusins. It is encoded by the nuclear gene *opa1* of 30 exons, where its expression is different depending on the tissue type. Eight different isoforms exist in humans and 5 different ones exist in mice, this is due to the alternative splicing of *opa1* exons 4, 4b, and 5b (Delettre et al. 2001; Ishihara et al. 2006). It is composed of an addressing sequence (mitochondrial targeting sequence MTS), a transmembrane domain TM, an alternative splicing domain AS, a coiled-coil structure CC, a GTPase domain, a middle domain, a pleckstrin homology domain (PH), and a GTPase effector domain GED (Lee and Yoon 2016) (Figure 26).

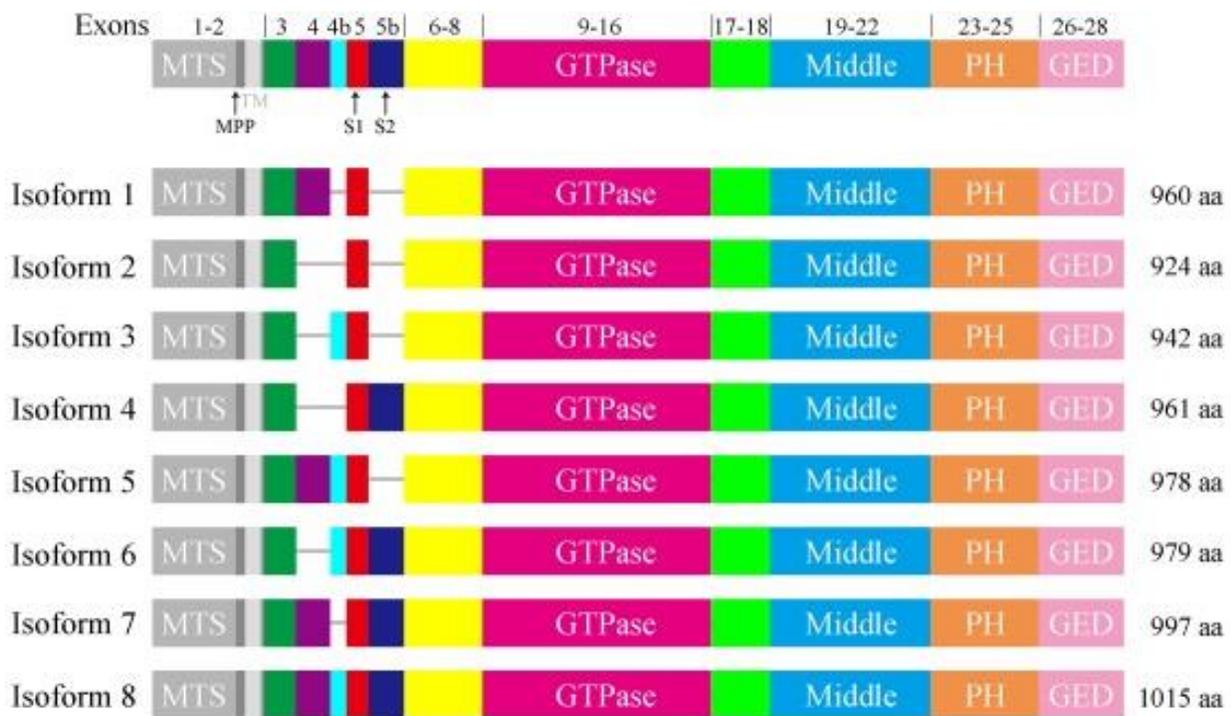


Figure 26 Human *OPA1* gene with the exons 4 and 5b, which are alternatively spliced to the eight isoforms shown. Number of exons and mitochondrial proteolytic cleavage sites including MMP, *OMA1* assigned by S1 and *YME1L* assigned by S2 (D. Li et al. 2019).

In the AS domain, there are the cleavage sites S1 and S2 where alternative splicing of exons 4, 4b, and 5b occurs to produce the different variants OPA1. In humans, the mRNA produced from the eight isoforms after AS splicing are targeted to the mitochondria through the MTS. Mitochondrial processing peptidase

(MPPs) cleaves the MTS to produce the long forms (l-forms) that will anchor the IMM. In contrast, OMA and YME1L proteases can cut about half of the l-forms to give rise to short forms (s-forms), except for the four isoforms of the exon 4b where all the 4b products are cleaved to s-forms, which are soluble in the IMS (D. Li et al. 2019). Remarkably, stress conditions that lead to mitochondrial dysfunction are almost attributed to a decrease in the expression of the long form of OPA1 (Adaniya et al. 2019).

Under normal conditions, the activity of YME1L increases in response to increased energy demands. In this situation, there is a mixture of short and long forms of OPA1, this will result in increased membrane potential and oxidative phosphorylation (Mishra et al. 2014). However, under stress conditions where there is a decrease in mitochondrial membrane potential, the activity of OMA1 is stimulated and thus more short forms are produced (Ishihara et al. 2006).

Figure 27 shows the eight isoforms of OPA1 with a mitochondrial leader sequence (MLS) that is cleaved by the MMPs. In (A), OPA1 isoforms have S2 (cleavage site for exon 5b) or S3 (cleavage site for exon 4b), are cleaved by YME1L (with a protease domain PD, and ATPase associated with diverse cellular activities AAA domain) to produce S-form of OPA1 (s-OPA1). Whereas isoforms lacking the exons 4b and 5b are not cleaved by YME1L, these produce only long L-forms of OPA1 (L-OPA1). However, in (B) the L-form which is not cleaved by YME1L remains exposed to OMA1 that cleaves in the S1 cleavage site of exon 5. Under normal conditions, OMA1 is inhibited by high mitochondrial membrane potential and high ATP production, and OMA1 is degraded by proteolysis. Several factors like loss of mitochondrial membrane potential or reduction in the ATP levels can lead to the activation of OMA1 and cleavage of L-OPA1 (Head et al. 2009).

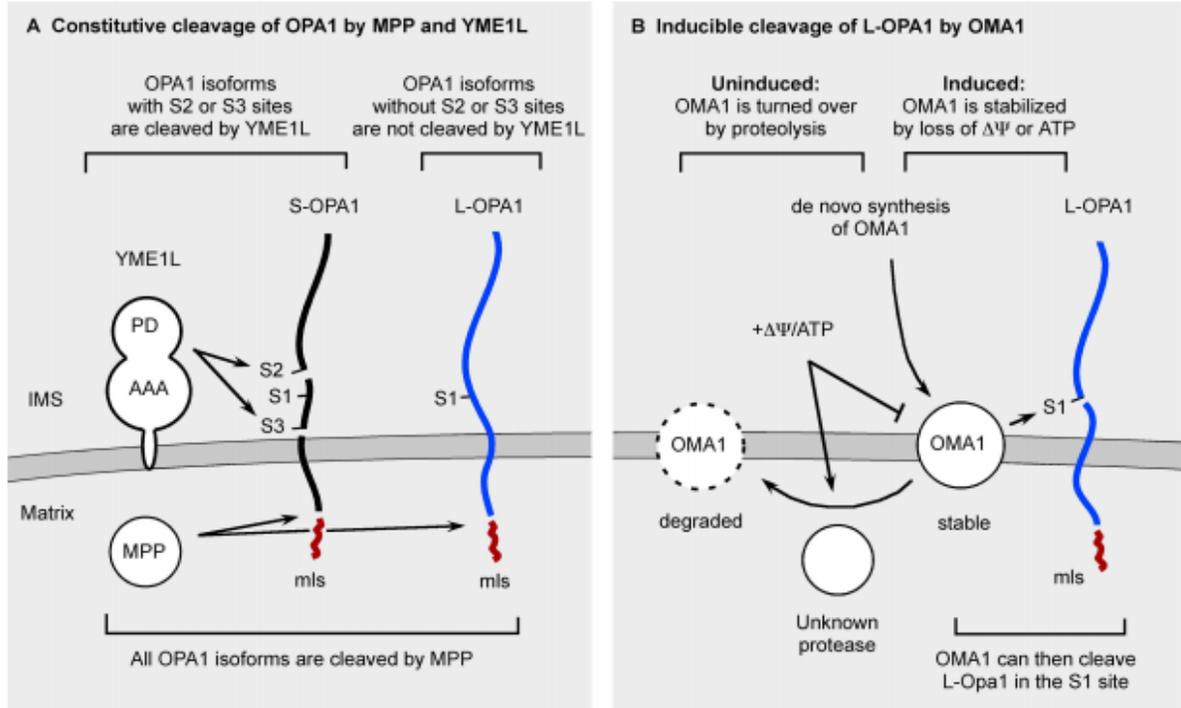


Figure 27 The different proteolytic cleavages of OPA1. Mitochondrial fusion requires both forms of OPA1 (a mixture of short and long forms). (A) Constitutive cleavage of OPA1 by MPP and YME1L and (B) the inducible cleavage of L-OPA1 by OMA1 (Head et al. 2009).

After its contact with the mitochondria, OPA1 can undergo some posttranslational modifications on specific residues to regulate its activity. These modifications include constitutive or inducible proteolytic cleavage, glycosylation, and acetylation. For instance, under stress conditions, a reduction in OPA1's GTPase activity due to its hyperacetylation at lysine residues 926 and 931 (Samant et al. 2014).

In addition to its role in mitochondrial fusion in cooperation with mitofusins, OPA1 maintains the cristae structure through the oligomerization of two L-OPA1 and one S-OPA1 that then acts with other protein complexes to alter cristae structure (Santarelli et al. 2015). During low energy substrate availability, cristae regulation is required to maintain mitochondrial activity and thus, OPA1 contributes to a role in OXPHOS indirectly through cristae regulation, as the mitochondrial respiratory chain complexes form supercomplexes localized in the cristae (Patten et al. 2014). Moreover, as the cristae are stabilized by OPA1 protein, it will help in preventing cell death through its proven role in the protection against cytochrome c release and ROS production (Varanita et al. 2015).

2.2.2. Mechanism of mitochondrial fusion

Three stages define mitochondrial fusion of the OMM, shown in Figure 28. The first stage is characterized by the anchoring of the two mitochondria to each other in trans through the HR2 domain of MFN1 and MFN2 with or without the aid of their GTPase domains exposed to the cytosol. GTP hydrolysis will lead to conformational change in the MFNs structures, this will increase the sites of contact of mitofusins with each mitochondrion and leads to mitochondrial docking, and this is considered as stage 2 in the mitochondrial fusion process. Finally, the outer mitochondrial membranes are pulled together after a GTPase-dependent power stroke, then subsequently the two membranes fuse.

After OMM fusion, OPA1 and Cardiolipin (CL) drive the IMM fusion where part of OPA1 is localized in the intermembrane space and resembles the MFNs structure, this will facilitate the interaction of OPA1 with mitofusins in the OMM. Then, a complex of OPA1-CL is formed to ensure the tethering of the two inner mitochondrial membranes. Then the mitochondrial content is fused after an OPA1-dependent GTP hydrolysis (Tilokani et al. 2018). In the absence of OPA1, only a superficial fusion of the OMMs with the absence of IMM fusions can occur. Consequently, the mitochondrial matrices are not mixed and the mitochondria lost their heterogeneity with a disturbance in normal metabolic activity of the mitochondria (Song et al. 2009).

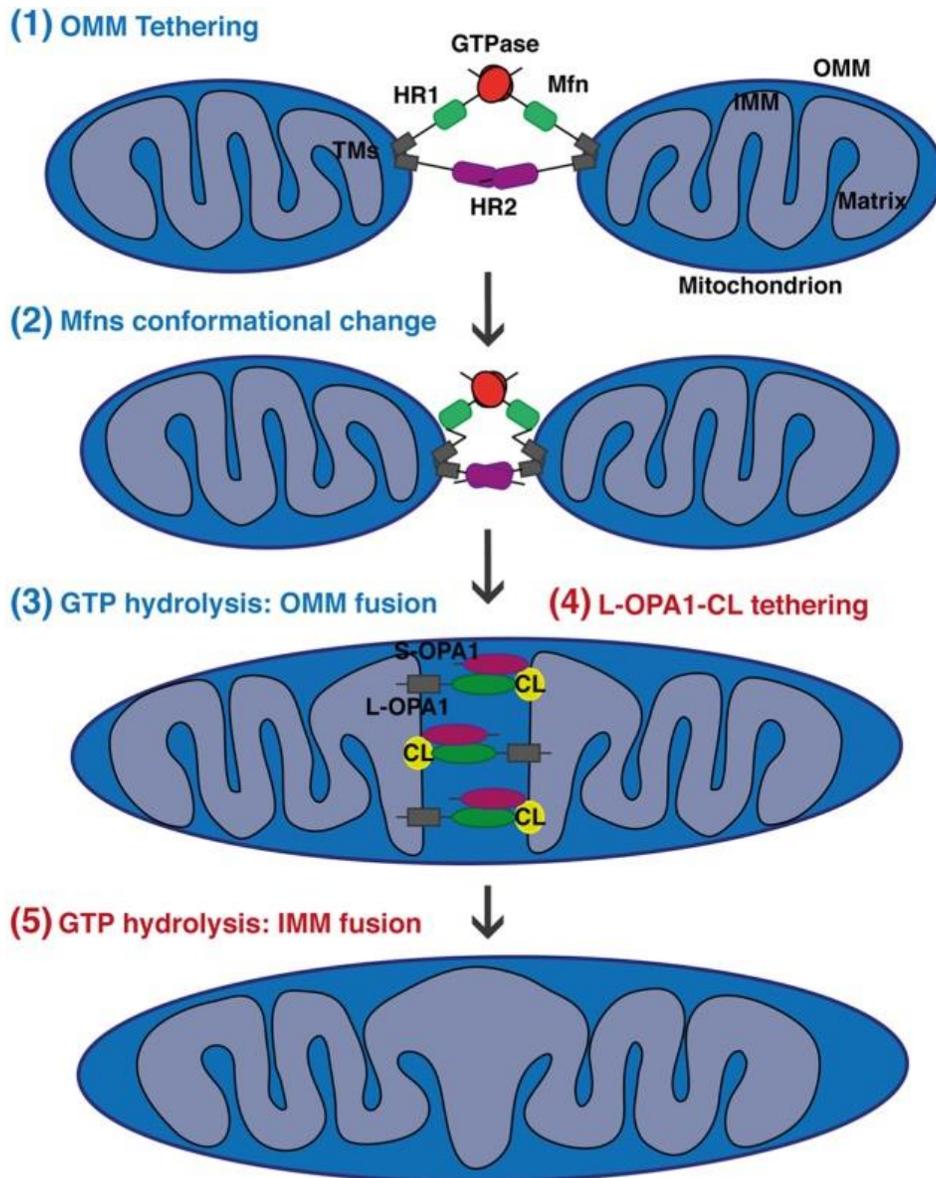


Figure 28 Mitochondrial fusion steps in mammals (Tilokani et al. 2018)

Lastly, the exact mechanism by which OPA1 fuses with the IMM together is still unknown. But, after mitochondrial fusion, all the components machinery remains associated with the inner and outer mitochondrial membranes but in a dissembled form (Tilokani et al. 2018).

2.3. Mitochondrial Fission

Mitochondrial fission is the symmetrical splitting of one large mitochondrion into two small mitochondria. A multistep process requires the recruitment of the GTPase Dynamin-related protein DRP1 to the mitochondria to mediate mitochondrial constriction. This process serves in controlling the number and size of the mitochondria. In mammals, mitochondrial fission is mediated mainly by GTPase Dynamin related

protein DRP1 (or DLP1) that binds to receptors on the mitochondrial membrane-like mitochondrial fission protein 1 FIS1, Mitochondrial Fission Factors MFFs, Mitochondrial Dynamics protein 49 MiD49, and Mitochondrial Dynamics protein 51 MiD51. Mitochondrial Dynamics proteins 49 and 51 are also known as mitochondrial elongation factors (MIEF) 1 and 2, respectively.

2.3.1. Mitochondrial Fission Proteins

Mitochondrial fission proteins are detailed below in their structure, regulation, and functions, where they are classified into DRP1, as it is the principal actor in mitochondrial fission process, and into DRP1 receptors for all the other factors acting in this process.

Dynamin Related Protein 1 (DRP1 or DLPI)

DRP1 is a member of dynamin superfamily proteins and encoded by the nuclear gene Dnm11 (dynamin 1 like). It exists as homo-oligomers where its activity depends on its oligomerization capability. Dimers reorganize DRP1 to remodel the mitochondrial membrane without inducing fission, however multimers of DRP1 induces mitochondrial fission through the its GTPase activity (Macdonald et al. 2014). It is an 80KDa cytosolic protein ubiquitously expressed but highly expressed in the brain, skeletal muscles and the heart (Smirnova et al. 1998).

In mammals, DRP1 consists of four different domains: highly conserved N-terminal GTP binding domain (G domain), middle domain, insert B domain, variable domain (VD), and a C-terminal GTPase Effector Domain (GED) (Lee and Yoon 2016). G domain and GED regulate the hydrolysis of ATP where alternative splicing may occur at the two sites, A-insert for GTPase domain and B-insert for the VD. As DRP1 doesn't have pleckstrin homology domains that allow it to anchor the outer mitochondrial membrane, its binding is mediated through DRP1 receptors on OMM (Figure 29).

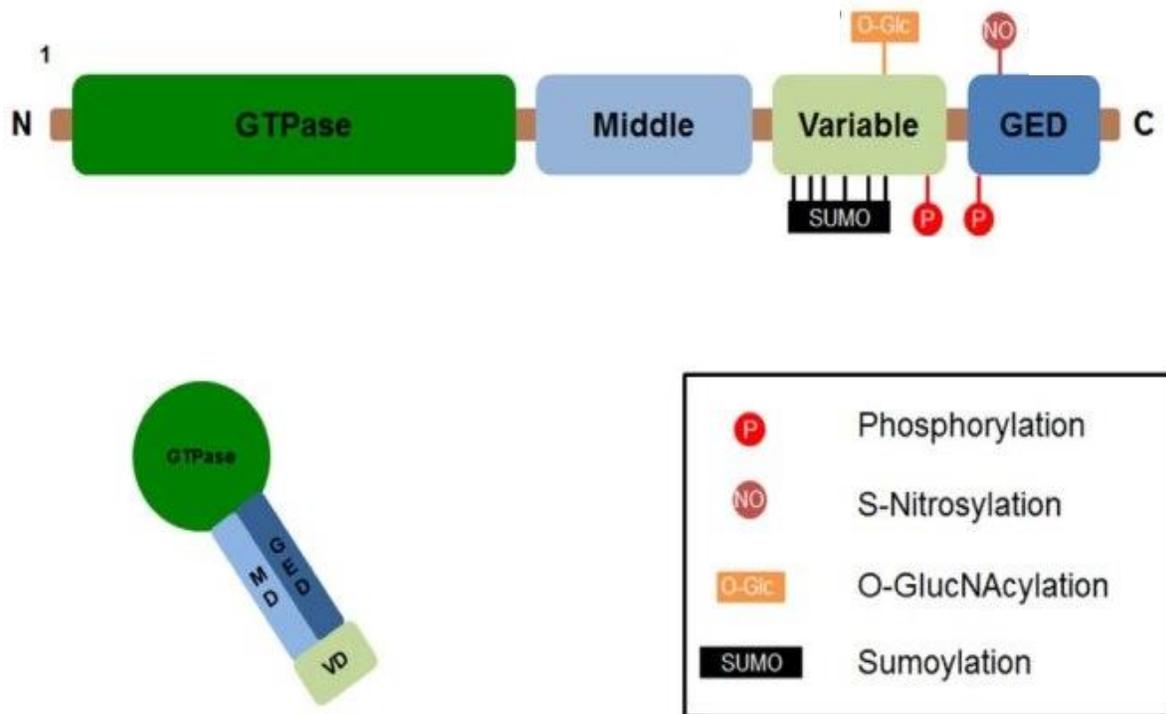


Figure 29 DRP1 protein structural domains with the sites of posttranslational modifications. The folded structure of DRP1 is also shown in this figure (B. Cho et al. 2013)

A variety of modifying enzymes can regulate DRP1 GTPase activity through posttranslational modification. The modifications are covalent and include protein phosphorylation, ubiquitination, and s-nitrosylation. However, it should be noted that posttranslational modifications of one site of DRP1 can affect another modifications in other sites (Chang and Blackstone 2010). All the posttranslational modifications acting on DRP1 are reversible, except for S-nitrosylation that is irreversible and leads to neurodegenerative alterations in DRP1. The reversibility in these modifications can reveal that these changes are important in the physiological control of mitochondrial functioning (B. Cho et al. 2013).

A phosphorylation in the serine 637 (S637) of human DRP1 by PKA can lead to a suppression of the translocation of DRP1 from the cytosol to the mitochondrial membrane by inhibiting its GTPase activity (Chang and Blackstone 2007). Whereas, after a calcium overload, calcineurin PP2B dephosphorylate DRP1 at S637 to promote mitochondrial fragmentation (Cereghetti et al. 2008). Also, mitochondrial fission could be promoted by DRP1 phosphorylation at serine 616 by Cdk1/cyclin B, this will allow DRP1 to translocate and interact with DRP1 receptors on the OMM to induce its fission (Marsboom et al. 2012). Moreover, an increase in mitochondrial fission after the addition of nitric oxide group to cysteine thiol through covalent bonding, a process known as S-nitrosylation (Figure 30) (D.-H. Cho et al. 2009)

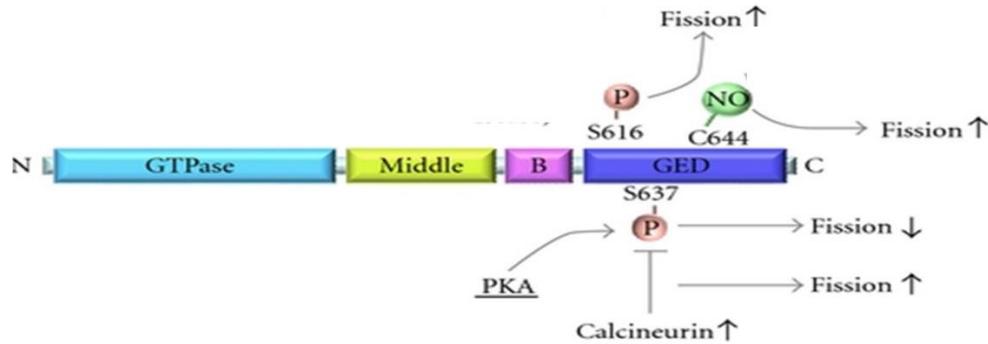


Figure 30 The effect of post-translational modifications on DRP1 structure and functioning (Otera and Mihara 2012)

Finally, other types of posttranslational modification can alter DRP1 functioning including ubiquitination, the addition of ubiquitin-protein to DRP1 middle domain by ubiquitin ligase enzymes (E1, E2, E3), will either increase or decrease mitochondrial fission to maintain normal cellular levels of DRP1. Similarly, small ubiquitin-like proteins called SUMO can bind to DRP1 and either inhibit (Chun Guo et al. 2013) or enhances its translocation to the mitochondria (Harder, Zunino, and McBride 2004), this post-translational modification is called SUMOylation.

To summarize, the different types of posttranslational modifications with their sites of action, the regulators involved, and their effects on mitochondrial fission are summarized in the table below (Table 1).

Modifications	Sites	Upstream regulators	Effects	References
Phosphorylation	S616	CDK1	Activation	Taguchi et al., 2007
		Erk1/2	Activation	Yu et al., 2011
		PKCδ	Activation	Qi et al., 2011
	S637	PKA	Inactivation	Chang and Blackstone, 2007; Cribbs and Strack, 2007b
		CaMK1α ROCK1	Activation	Han et al., 2008a Wang et al., 2012c
Dephosphorylation	S637	Calcineurin (PP2B)	Activation	Cereghetti et al., 2008
S-nitrosylation	C644	Nitric oxide	Activation	Cho et al., 2009
SUMOylation	Multi-sites in variable domain	MAPL	Activation	Braschi et al., 2009
DeSUMOylation		SEN5	Inactivation	Zunino et al., 2007b
Ubiquitination		MARCH5	Inactivation	Nakamura et al., 2006a; Itonashi et al., 2006b
		Parkin	Inactivation	Wang et al., 2011b
O-GlcNAcylation	T585, T586	O-GlcNAc-transferase	Activation	Gawłowski et al., 2012b

Table 1 The possible posttranslational modifications of DRP1 (B. Cho et al. 2013)

DRP1 receptors

After the recruitment of DRP1 to the outer mitochondrial membrane due to exposure to a mitochondrial fission stimulus. It binds to the mitochondrial membrane through protein receptors embedded in the OMM to form the fission complex. The first known receptor on the OMM for the recruitment of DRP1 was identified in yeast and it is called Fis1. Also, Fis1 was first described to be associated with the two adapter proteins Mdv1 and Caf4 (Tieu et al. 2002). However, in mammals, Mdv1 and Caf4 homologs are not identified and DRP1 was shown to interact with Fis1 to induce mitochondrial fission.

Fis1 is a mitochondrial protein with a mass of 17 KDa and it is evenly distributed along the outer mitochondrial membrane. Mitochondrial fission was thought to be only induced through the interaction of DRP1 with Fis1, however, Drp1 can be recruited to the mitochondria in a Fis1-independent manner. Although under physiological conditions Fis1 knockout doesn't lead to any morphological change in the mitochondrial network, it has been identified to have a role in stress-induced fission or autophagy (Shen et al. 2014). In mammalian cells, Fis1 knockout did not cause a deficiency in mitochondrial fission and another factor was compensating for the loss of Fis1, this factor is the mitochondrial fission factor Mff (Otera et al. 2010). Therefore, after the posttranslational modification that induces the translocation of DRP1 to the mitochondria, DRP1 forms complexes with Mff on the OMM to induce mitochondrial fission. Afterward, two factors were identified to act in the mitochondrial fission process; these factors were MiD49 and MiD51 (also referred to as MIEF2 and MIEF1) (Palmer et al. 2011). These seem to recruit the DRP1s, which are phosphorylated on serine 637 to the outer membranes, so they form complexes with DRP1 and block DRP1 from binding to the Fis1 and Mffs, or they sequester Fis1 to block its binding to DRP1. In contrast, after the overexpression of Fis1, the inhibitory effect of MIEF1/MiD51 on DRP1 was reduced, and thus mitochondrial fission was activated (Figure 31) (Dikov and Reichert 2011).

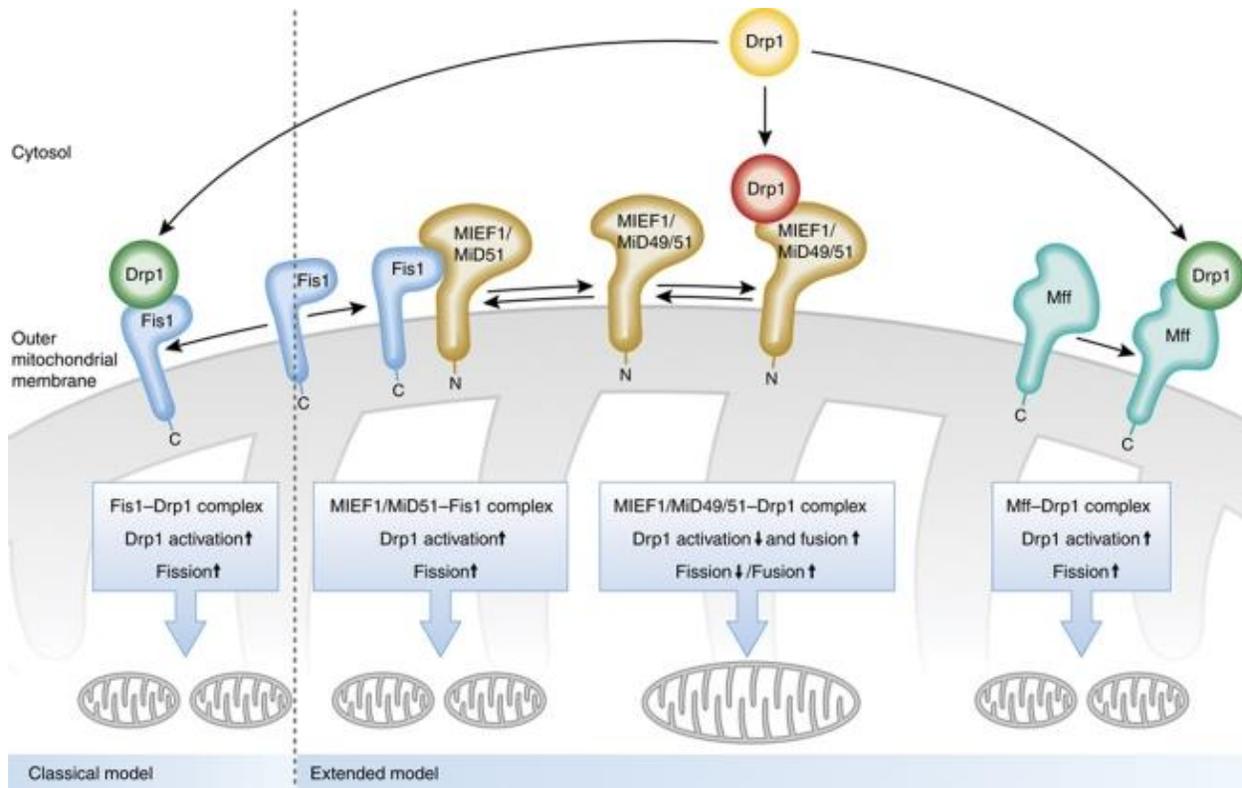


Figure 31 The different complexes regulating mitochondrial fission in mammals. On the left, the classical model of mitochondrial fission (the earliest model) where DRP1 interacts only with Fis1, and on the right, an extended model where Fis1 is not the only DRP1 receptor. Thus, DRP1 can bind to various receptors to either inhibit or activate mitochondrial fission (Dikov and Reichert 2011)

2.3.2. Mechanism of mitochondrial fission

Mitochondrial fission occurs with the aid of another organelle, the endoplasmic reticulum ER. As a pre-step in this process, when the mitochondria duplicated its DNA by the replication process, the mtDNA marks the site for the recruitment of the ER. When the ER encircles the mitochondrion at the sites of fission, it stimulates actin polymerization providing the necessary forces for the preconstruction of the mitochondria. While DRP1 monomers are in equal balance between cytosol and mitochondria, the ER provides the site for the dynamic recruitment of DRP1 from the cytosol to the mitochondria following various signals and post-translational modifications. DRP1 can form spiral-shaped complexes on the OMM in contact with the ER, through its GED domain in a calcium-dependent process (Haun, Nakamura, and Lipton 2013). Many internal factors in the matrix are involved in the recruitment of actin and myosin to the fission sites where they are essential for mitochondrial constriction. A conformational change in DRP1 enhances the formation of a ring-like structure due to the hydrolysis of GTP. At this time, DRP1 can interact with its receptors including Fis1, Mffs, and other receptors. In the last step of mitochondrial fission, a GTPase protein called Dnm2, assemble on the neck of the constricted site to terminate the scission of the

mitochondrial membrane and form two daughter mitochondria (Figure 32) (Lee and Yoon 2016; Kraus and Ryan 2017).

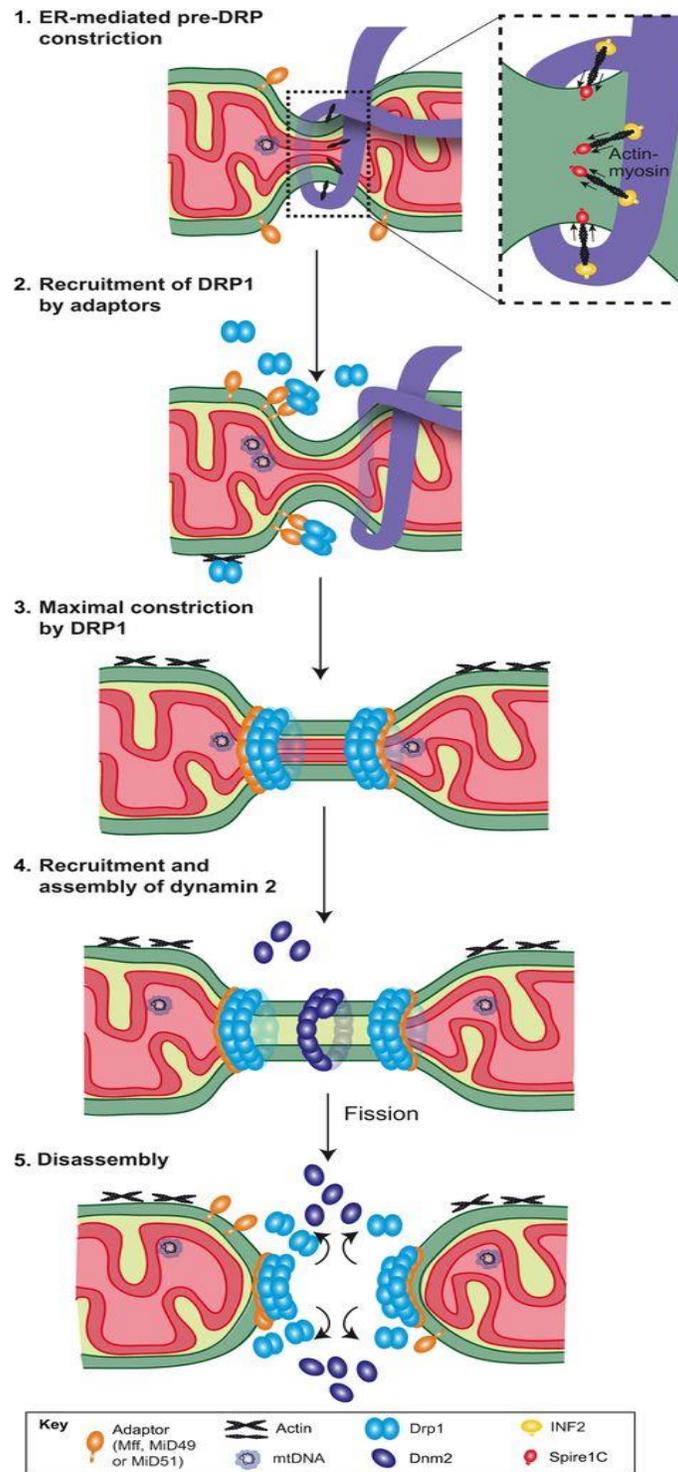


Figure 32 Mechanism of mitochondrial fission in mammals (Kraus and Ryan 2017)

3. Shear stress and endothelial mitochondrial dynamics

The vascular system is aligned with endothelial cells that play a major role in vascular homeostasis in the bloodstream. Different endothelial beds contain a variable content of mitochondria depending on the cell demands but the number of mitochondria in endothelial cells is low compared to other cell types. Although in most of the cells the primary role of the mitochondria is to produce ATP via OXPHOS, vascular endothelial cells do not rely on mitochondria for the production of their ATP and they supply their energy demands by anaerobic glycolysis. However, the mitochondria in endothelial cells take part of shear stress mechano-transduction into biochemical signals, altering many cellular processes and functions. Also, many regulatory processes in endothelial cells are maintained through the mitochondria, which are considered as the efficient metabolic regulators of the cells to allow cell proliferation, trigger apoptosis, and transduce biochemical signals.

Mitochondria play an important role in the propagation and transduction of calcium signals, regulating the mechanotransduction process. In response to shear stress, endothelial cells release intracellular calcium levels from the ER stores. An increase in the levels of intracellular calcium senses the uptake of intracellular calcium by the mitochondria through the mitochondrial calcium uniporter MCU, this leads to an overload in the mitochondrial calcium levels sensing the opening of the mPTP. The high cellular localization of calcium deactivates IP3R closing the calcium efflux from ER and opening the MCU for the mitochondrial calcium uptake, thus reforming the release of intracellular ER calcium waves through mNCKX and altering the response of endothelial cells to shear stress (Scheitlin et al. 2016). Under physiological conditions, mitochondrial dynamics highly maintain mitochondrial quality through the regulation of mitochondrial biogenesis and degradation. However, under inflammatory conditions, a reduction in the levels of mitochondrial biogenesis was observed. This reduction was associated with a decrease in the levels of mitochondrial membrane potential ($\Delta\Psi_m$) that results in mitochondrial dysfunction (Dada and Sznajder 2011).

Mitochondrial biogenesis is implicated in the endothelial redox state (Davidson Sean M. and Duchon Michael R. 2007), where PGC1- α induces ROS detoxifying enzymes that scavenge free radicals (ROS and RNS) (St-Pierre et al. 2006). In general, in response to shear stress endothelial cells secrete NO and prostacyclin in a dose dependent manner, these latter relaxes smooth muscle cells and decreases the vascular tone in order to maintain a stable shear stress. Laminar flow has been shown to increase the expression of SIRT-1 and AMPK protein synergistically leading to an increase in eNOS-derived NO bioavailability in response to shear stress. Thus, shear stress enhances mitochondrial biogenesis protecting endothelial cells from oxidative stress (Z. Chen et al. 2010). Besides, Wu et al have shown that endothelial cells an increase

in mitochondrial ROS with an increase in the mitochondrial membrane potential of these cells (L.-H. Wu et al. 2018).

However, mitochondria are dynamic organelles altering their inner and outer membranes through fission and fusion mechanisms. In a response to shear stress, the mitochondria in endothelial cells (ECs) continuously undergo fusion and fission mechanisms. Romero et al. have shown in a recent study that an intracellular signaling pathway was activated by LSS that modulate mitochondrial dynamics and function. In an early stage of response to shear stress, an increase in the free cytosolic calcium concentration induces an alteration in the mitochondrial dynamics of ECs by enhancing mitochondrial fission through the activation of mitochondrial fission protein DRP1 and translocating it into the mitochondria (Figure 27). Thus, a decrease in the mitochondrial respiration rate with an increment of mitochondrial membrane potential associated with an increase in the ROS production and consequently PRX3 activation, as first line antioxidant signaling adapters to shear stress (Bretón-Romero et al. 2014).

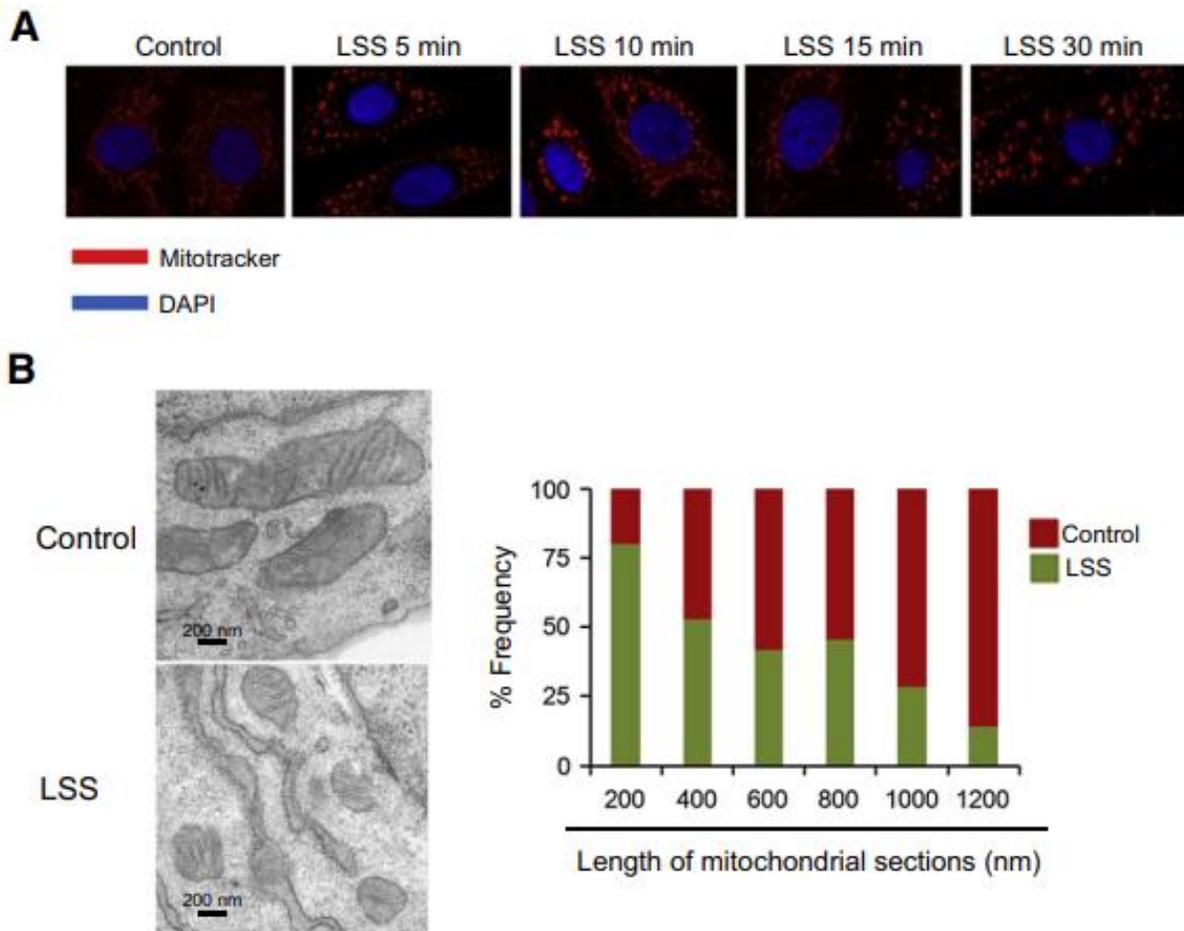


Figure 33 The effect of laminar shear stress LSS on mitochondrial network. (A) Mitotracker red staining shows that the mitochondrial network is highly fragmented after endothelial cells exposure to shear stress LSS for 5, 10, 15 and 30 mins. These results was further verified through (B) electron microscopy images for the mitochondrial structure

after applying shear stress for 15 mins, where the bar graph shows that most of the ECs exposed to shear stress have the smallest length of mitochondrial sections (Bretón-Romero et al. 2014).

On the other hand, Wu et al. have shown in 2018 that the DRP1 translocation to the mitochondria after the exposure of ECs to shear stress was reduced. This reduction was referred to the decrease in the DRP1 phosphorylation at ser 616 and an increase in the one at ser 637 that inhibits DRP1 translocation from the cytosol to the mitochondria. Also, these cells (Human Umbilical Vein Endothelial Cells) under flow of 12 dynes/cm² have shown an increase in mitochondrial fusion. This fusion appears in the form of an increase in the levels of Mfn2 and OPA1, with a decrease in Fis1 levels. The increase in YME1L protease levels and the high levels of S-OPA1 and L-OPA1 can confirm these results. Thus, in contrast to the study of Breton-Romero et al. shown above, shear stress alters mitochondrial dynamics by shifting more the balance more toward mitochondrial fusion and not fission (L.-H. Wu et al. 2018).

Moreover, cells exposed to inflammatory substances like TNF α can lead to mitochondrial dysfunction through the decrease in mitochondrial biogenesis and $\Delta\Psi_m$ (Dada and Sznajder 2011). After the treatment of cells with only TNF α , the levels of mitochondrial fusion proteins decreased but in the cells treated with TNF α and then subjected to shear stress, an attenuation to the effect of TNF α on the mitochondria by inhibiting its effect on mitochondrial fragmentation have been observed. Also, after shear exposure, the cells showed a decrease in the levels of DRP1 associated to the mitochondria compared to the cells only treated with TNF α . Therefore, shear stress enhances mitochondrial fusion, reduces mitochondrial fission, and mitigates the mitochondrial fission and the mitochondrial impairments induced by TNF α (Figure 34) (L.-H. Wu et al. 2018).

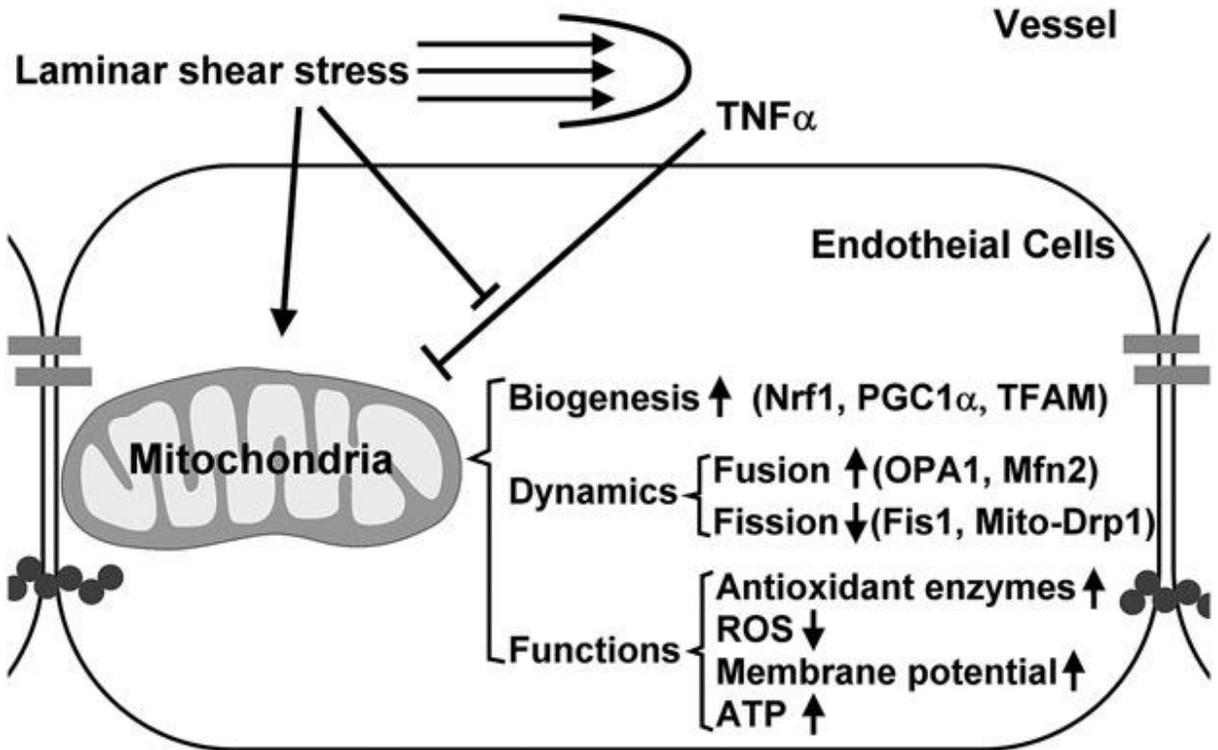


Figure 34 Effect of shear stress on mitochondrial dynamics and inflammatory response of the mitochondria (L.-H. Wu et al. 2018).

As shear stress is affecting mitochondrial dynamics and function, the levels of mitochondrial ATP were monitored in a study exposing endothelial cells to shear stress and they have shown that upon exposure of the cells to flow, the levels of mitochondrial ATP were directly dependent on the intensity of shear stress. Shear stress-induced ATP generation or ATP release was not affected after the inhibition of the cytosolic glycolysis confirming that the mitochondria is the origin of ATP released by shear stress (Yamamoto, Imamura, and Ando 2018).

In conclusion, there are no compatible results between the two studies on the effect of shear stress on the mitochondrial dynamics of endothelial cells and thus, we have decided to study this effect invitro and in vivo by using mice models. Opal and Drp1 knocked out mouse models have been used in our study to better understand the effect of mitochondrial dynamics in the response of endothelial cells to shear stress.

4. Mitochondrial Dynamics and cardiovascular pathophysiology

As we have explained before, the balance in mitochondrial dynamics is indispensable for cell homeostasis. It is an essential mechanism to maintain vascular structure and function. Indeed, emerging data have suggested that changes in mitochondrial morphology, through fusion and fission activity, can lead to several cardiovascular pathologies including cardiac hypertrophy, heart failure, myocardial infarction,

hypertension, etc. (Figure 35). Also, this effect is reversible as also some cardiovascular diseases lead to a change in mitochondrial dynamics balance in these cells. For instance, in diabetic cardiomyopathy patients, an enhanced mitochondrial fission is observed with an increase in Fis1 levels and a decrease in mitochondrial OPA1 and Mfn1 levels (Montaigne et al. 2014).

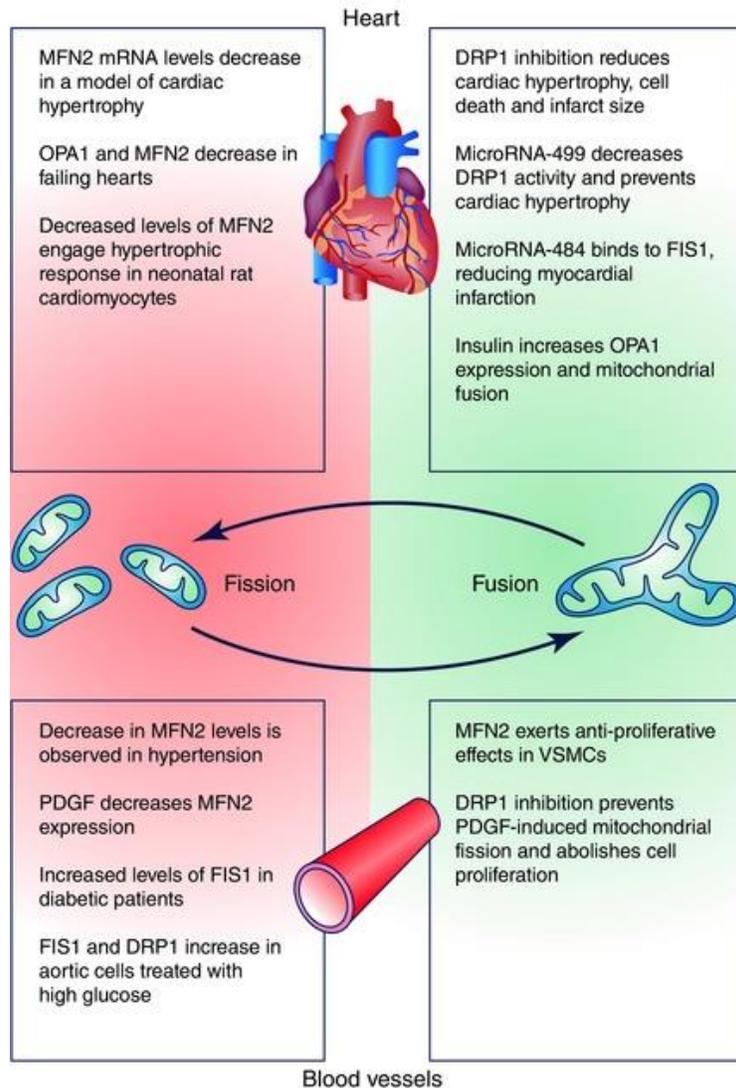


Figure 35 The different alterations in mitochondrial dynamics and cardiovascular diseases (Montaigne et al. 2014).

However, as our project is concerned more with endothelial dysfunctions and shear stress, we will focus on the effect of mitochondrial dynamics in atherosclerosis.

Mostly, in vascular diseases, the mitochondria is implicated in pathologies through the increase in the levels of mitochondrial ROS generation (Y.-E. Cho et al. 2013; Ying Wang and Tabas 2014). But, mitochondrial DNA damage by some stress promotes directly atherogenesis in both human aortic specimens and mouse models. For instance, ApoE^{-/-} mice showed an early increase in mtDNA damage

with the absence of the mitochondrial antioxidant enzyme MnSOD, which results in accelerated atherogenesis (Ballinger et al. 2002). The vascular wall is affected by the increase of ROS levels where it causes endothelial dysfunction at the level of the tunica interna, along with an increase in the proliferation and apoptosis of SMCs and macrophages. As a result of these alterations, atherosclerotic lesions are developed with the risk of plaque rupture in the vessels (Madamanchi and Runge 2007). This result was compatible with the results of mitochondrial dynamics proteins of ECs from type 2 diabetes mellitus T2Dm patients, where they showed an increase in mitochondrial fission in venous ECs. This result was in agreement with the results obtained after the incubation of human aortic endothelial cells HAEC in a high glucose medium where they showed increase in ion mitochondrial fission by the increase in both DRP1 and Fis1 proteins. Although elevated ROS levels were observed with the alterations in mitochondrial dynamics, these cells lost their high glucose-induced mitochondrial dynamics alterations without any increase in mitochondrial ROS production (Shenouda et al. 2011). Also, retinol binding protein 4 RBP4, an adipokine usually found at high levels in the blood samples from metabolic syndromes, ECs treated with RBP4 showed an increase in mitochondrial fission accompanied with a reduction in mitochondrial fusion (J. Wang et al. 2015).

At the level of vascular SMCs, an increase in their proliferation level is a marker of atherosclerosis, where their proliferation is triggered by PDGF (Perez et al. 2010). After the exposure of vSMCs to PDGF, Salabei and Hill have observed a 50% reduction in Mfn2 levels with an enhancement in mitochondrial fragmentation (Salabei and Hill 2013).

As a conclusion, mitochondrial dynamics are shifted more toward mitochondrial fission in most of cardiovascular diseases. For that, emerging studies are targeting mitochondrial dynamics as a valid therapeutic strategy in cardiovascular diseases. For example, mdivi1, a molecule used for mitochondrial fission inhibition is an emerging molecule in the treatment of cancer. This is due also to the increase in mitochondrial fusion processes after the reduction in mitochondrial fission (Dai et al. 2020).

Aim of the thesis

In our laboratory, projects are developed to study cardiovascular and mitochondrial physiopathology. Different topics are studied that aims for finding cardioprotective strategies. This is the main goal of our topic, which will be detailed in this thesis manuscript.

Mitochondrial dynamics proteins play several roles other than mitochondrial fission and fusion. Mostly, these roles are achieved through their alterations in the functioning of the mitochondria and in the response to several stress activating the cells. Thus, the alteration in the balance of mitochondrial dynamics could contribute to the regulation of vascular functioning and reactivity.

However, the potential role of OPA1 and DRP1 in the arterial responses to shear stress is not yet well understood. Although in 2014, Bréton-Romero et al. showed that laminar shear stress has increased mitochondrial fission in endothelial cells with an increased level of mitochondrial DRP1, another study in 2018 by Wu et al. has shown that endothelial cells subjected to laminar shear stress increased their levels of mitochondrial fusion with a reduction in the levels of DRP1 translocated to the mitochondria (Bréton-Romero et al. 2014; L.-H. Wu et al. 2018). These two contradictory results were not verified in-vivo through animal models. In this study, we investigated the role of OPA1 and DRP1 in the arterial response to shear stress and thus, how these could modulate the functioning of endothelial cells. Furthermore, as endothelial dysfunction occurs in an early stage of atherosclerosis, we aimed to study the role of OPA1 and DRP1 in atherosclerotic lipid deposition using OPA1 and DRP1 knocked out mouse models (*Opa1*^{+/-} & *Drp1*^{+/-}). For these mice to develop atherosclerotic plaque in their arteries we used low density lipoprotein receptor *ldlr* null mice (*ldlr*^{-/-}).

Materials & Methods

Invivo Experiments

1. Mouse Models

We used the *Opal* knocked out mice carrying the recurrent *OPAI* c.2708_2711delTTAG mutation, described in patients by deleting the four conserved TTAG base pairs in exon 27 of the murine *Opal* gene and integrating by homologous recombination the construct with a neomycin cassette at the *Opal* gene locus in embryonic stem cells. Recombinant embryonic stem cells (129/SvPas strain) were injected into embryonic blastocysts of C57Bl6/J at day 3.5, leading to genetically modified chimaeras. Finally, the F1 breeding of chimaeras with creatine-expressing mice resulted in the elimination of the neomycin cassette, leading to the constitutive *Opal* knock-in mice (GenOway). C57BL/6J deleted for the gene coding for the LDL receptor *Ldlr* *-/-* mice were crossed with mice carrying the c.2708_2711delTTAG mutation of the *Opal* gene (*Opal* +/-) and WT mice of (*Opal*+/+) gene. The new generations obtained are all *Ldlr* *-/-* *Opal*+/+ or *Ldlr* *-/-* *Opal* +/- where *Ldlr* *-/-* *Opal* *-/-* were embryonically dead at day 13.5 post coitum.

Heterozygous *Drp1* knockout (*Drp1* +/-) mice were obtained as previously described (Wakabayashi et al. 2009) from Professor Hiromi Sesaki at the Johns Hopkins University. They were generated by the insertion of a neomycin-resistant marker flanked by flipase (Flp) recombinase target and loxP sites next to exons 3 and 5, which are located in an essential GTPase domain. The target region was ~3.3 kb and included exons 3–5. The targeting vector was transfected into C57BL/6-129/SvEv ES cells by electroporation. G418-resistant colonies were screened by PCR. Targeted ES cells were injected into C57BL/6 blastocysts to create chimeric mice. To create the null *Drp1* allele, they crossed Flox-neo mice to a transgenic strain that expresses Cre recombinase from the EIIa promoter and confirmed loxP recombination by PCR. In addition, upon loxP recombination, a stop codon was generated immediately after exon 2 as a result of a frame shift. To generate the conditional allele, Flox-neo mice were crossed to a transgenic strain that ubiquitously expresses Flp recombinase and confirmed Flp recombinase target recombination by PCR. They bred these strains to a wild-type strain and isolated mice heterozygous for the null or conditional allele but not Cre or Flp recombinase. C57BL/6J deleted for the gene coding for the LDL receptor *Ldlr* *-/-* mice were crossed with mice carrying the *Drp1* +/- mutation (*Drp1* +/- heterozygous mice express a partially reduced level of DRP1 ~75% of wild-type level). All the new generations obtained are *Ldlr* *-/-* *Drp1*+/+ or *Ldlr* *-/-* *Drp1* +/-, where *Ldlr* *-/-* *Drp1* *-/-* were embryonically dead at day 11.5 during embryogenesis.

Mice were kept in the “*Service Commun d’Animalerie Hospitalo Universitaire*” (SCAHU). Housing rooms were equipped with standard light cycles (12h light/12h dark), with food and water available *ad libitum*.

Part of the animals were placed on a cholesterol-enriched atherogenic diet (1.25% cholesterol, 6% fat, no cholate, TD96335, Harlan Teklad, Wisconsin), High Fat Diet (HFD - U8959 Version 0022210, SAFE)

treatment was left for 4 months. In accordance with the ethical rules, some studies have been performed on cells. The procedures performed on animals are validated by the ethics committee (CEEA Pays de la Loire) and the Ministry of Research (N°APAFIS 3577 Ldlr/Opa1),

In all protocols, littermate mice were used as control.

2. Mice Ovariectomy

Ovariectomy was performed in some female mice as previously described (Tarhouni K. et al. 2013). Three-month-old female mice were ovariectomized (OVX) for 14 days and then submitted to HFD for 4 months. To assure the succession of OVX, the uterus weight was measured after mice dissection. In each protocol, animals were anesthetized with isoflurane (2.5%) and treated with 2.5 μ L/g buprenorphine (Temgesic, 0.02 mg/ml, s.c.) before and after surgery.

3. Flow Mediated Outward Remodeling

2 months old mice anesthetized [isoflurane (2.5%)] and submitted to surgery to increase blood flow in one mesenteric artery. Three consecutive first-order arteries were used. Ligatures were applied to second-order branches. The artery located between the 2 ligated arteries was designated as a high-flow (HF) artery, Arteries located at a distance from the ligated arteries were used as a control (normal flow, NF), and the arteries ligated were used as low flow (LF) arteries. Mice were treated either with E2 (20 μ g/kg per day, osmotic minipump, n=12) or with solvent (ethanol 5% in 0.9 g/L NaCl, n=12). In each protocol, animals were treated with 2.5 μ L/g buprenorphine (Temgesic, 0.02 mg/ml) before and after surgery.

The protocol conformed with European Community standards on the care and use of laboratory animals (Authorization No. 00577) and with National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). The protocol was approved by the ethical committee (Permit No. CEEA PdL 2008.10). Fourteen days after surgery, mesenteric arteries were quickly removed and placed in liquid nitrogen for qRtPCR experiments.

All procedures involving experimental animals 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. The investigation conforms to the directive 2010/63/EU of the European parliament.

Ex-vivo experiments

4. Flow-mediated dilatation in mesenteric arteries *in vitro*

(Arteriography)

Arterial segments, approximately 200 μm in internal diameter, were cannulated at both ends on glass micro-cannulae and mounted in a video-monitored perfusion system (Living System, LSI, Burlington, VT) (Iglarz, Lévy, and Henrion 1998). The artery segment was bathed in a 5 ml organ bath containing PSS of the following composition (mmol/L): 130.0, NaCl; 15.0, NaHCO_3 ; 3.7, KCl; 1.6, CaCl_2 ; 1.2, MgSO_4 and 11.0, glucose (pH 7.4, pO_2 160 mmHg and pCO_2 37 mmHg). Perfusion of the artery was obtained with 2 peristaltic pumps, one controlling flow rate and one under the control of a pressure-servo control system. Pressure at both ends of the artery segment was monitored using pressure transducers.

Arterial contractility was assessed with precontraction with KCl (80 mmol/L) and phenylephrine 1 $\mu\text{mol/L}$. Endothelium-mediated dilation with acetylcholine (1 $\mu\text{mol/L}$) after precontraction with phenylephrine (50% of maximum contraction). Pressure was then set at 75 mmHg and flow (3 to 50 μl per min) could be generated through the distal pipette with a peristaltic pump in order to determine flow-mediated dilatation (Henrion et al. 1997).

Arteries were then bathed in a Ca^{2+} -free PSS containing ethylene-bis-(oxyethylenenitrolo) tetra-acetic acid (2 mmol/L) and sodium nitroprusside (10 $\mu\text{mol/L}$). Pressure was then increased by step from 10 to 125 mmHg, in the absence of flow, in order to determine the passive arterial diameter and passive mechanical properties of the arterial wall (wall thickness, cross-section area) as previously described (Bouvet et al. 2007).

5. Myography experiments

Segments of aorta and mesenteric resistance arteries (2mm long) were mounted on a wire-myograph. Two tungsten wires (40 μm diameter) were inserted into the lumen of the arteries and respectively fixed to a force transducer and a micrometer. Arteries were bathed in a PSS as described above. Wall tension was applied as described previously. After a 45 min equilibration period, artery viability was tested using a potassium rich PSS (80 mmol/L). Then, cumulative concentration-response curve to phenylephrine (1 nmol/L to 10 $\mu\text{mol/L}$) was performed. After washout, the arteries were pre-contracted with phenylephrine to a level approximately equivalent to 50% of the maximal response. Once a plateau was reached, cumulative concentration-response curve to acetylcholine (1 nmol/L to 10 $\mu\text{mol/L}$) was generated. After washout, the arteries were precontracted with phenylephrine (10 $\mu\text{mol/L}$). In order to test the NO sensitivity of the

vascular smooth muscle cells, a cumulative concentration-response curve to a NO donor, sodium nitroprusside (SNP) (1 nmol/L to 3 μ mol/L) was then performed.

6. Analysis of atherosclerotic lesions

Ten-week-old male and female mice (*Ldlr*^{-/-} mice crossed with *Opa1*^{+/-} or *Drp1*^{+/-} mice) were submitted to a hypercholesterolemic atherogenic diet (1.25% cholesterol, 6% fat, no cholate, TD96335, Harlan Teklad). After 4 months from western diet treatment (U8959P Version 0022, SAFE), 8 months old mice were anesthetized with isoflurane (2.5%), blood was collected in the aorta and then tissues were collected for analysis. The blood samples were collected in a heparin tube. The plasma is then recovered after centrifugation (20 min, 4 °C, 1500 rpf) to perform biochemical analysis: cholesterol level, triglycerides, HDL, LDL (carried out on the technical platform of the Centre Hospitalier Universitaire CHU d'Angers). The perigonadic fat and the liver were removed and weighed. In addition, the aortas were excised from the carotids to the iliac bifurcations with the heart. The aortic sinus is conserved in OCT medium (Tissue-Tek®, Miles, Inc.). The aorta is opened longitudinally and then placed in successive staining stages: Oil red O (sigma O0625), isopropanol 60%, and distilled water then photographed by the Live Video Output Leica IC80 HD. Finally, the surface area of the plates is quantified by the Image J software.

7. qRT-PCR in arteries

Ldlr^{-/-} mice were used for the study of the expression of the mitochondrial Dynamics genes in function of the flow. After sacrificing mice, the aorta was preleved from the carotids to the iliac bifurcations, the mesenteric arteries were also preleved. The aortic arch, ascending aorta (greater curvature of the aortic arch), thoracic aorta, abdominal aorta, carotids and the superior mesenteric arteries were placed in RNA-later all night at 4°C and then frozen at -20 °C. The samples are grinded and then solubilized in 360 μ l of QIAzol Lysis Reagent (QIAGEN). After extraction using the RNeasy Mini Kit (QIAGEN), the RNAs are stored at -20 °C until use. qPCR was then carried out in the same way as on the cells (part 13). The oligonucleotides used are listed in the Table 2.

In-vitro Experiments

8. Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Lonza (Walkersville, MD) and cultivated in endothelial cell growth medium-2 (EGM-2) (Lonza) supplemented with the EGM™ -2 SingleQuots kit (Lonza), at 37°C and 5% CO₂. Cells from passages 1 to 5 were used for experiments.

For orbital shaker experiments, MS1 (MILE SVEN1) cell line was used, an endothelial C57BL6 cell line obtained from the islets of Langerhans (ATCC®CRL2279™, France). Cells were maintained in a DMEM culture medium (Lonza) supplemented with 5% BSA, 1% Glutamine and 1% of Penicillin/Dihydrostreptomycin. The cells were maintained in a humid atmosphere of 37°C with 5% CO₂ (Carbon Dioxide).

9. RNA Interference

RNA interference experiments were performed by transfection of 50 nM of a silencer negative control silencer negative control (cat# AM4611 50µM) and a pre-validated OPA1 silencer select pre-designed siRNA against *OPA1* (Ambion ID s9851), and *DNM1L* genes (Ambion ID s19559) using the Lipofectamine™ transfection reagent (Invitrogen cat# 18324012), according the manufacturer's instructions.

HUVECs were seeded in a density of 750,000 cell/well in a 6 well plate. The lipofectamine and the siRNA were diluted in a reduced serum Opti-MEM (Gibco, Grand Island, New York). The diluted lipofectamine and siRNA were mixed in a 1:1 volume ratio and the mixture was incubated for 30 minutes at room temperature. Then, 250µL of the mixture was added to each well of the 6 well plate with 500µL of EBMTM-2 Endothelial Cell Growth Basal Medium-2 from Lonza (Walkersville, MD), and kept for 1 hour. 1 ml of EGM-2 medium was re-added with the addition of the same volume of EGM-2 after 48 hours.

For MS1 cells, cells were seeded in a 12 well plate with a density of 50,000 cell/well. The Lipofectamine and siRNA was diluted in a medium of transfection (DMEM supplemented with 5% SVF and 1% Glutamine, without antibiotics). The Lipofectamine diluted was added with siRNA in a 1:1 volume ratio and then incubated for 30 minutes at room temperature. 150µL of the mixture were added per well and incubated with the cells for 48 hours.

To verify siRNA efficiency, Western blot assay of OPA1 and DRP1 was carried out. Transfected cells were trypsinized, washed three times in ice-cold PBS, and harvested using 0.1% Triton X-100.

10. Shear stress application

The IBIDI pump system (Fluidic Unit: Cat.No. 10903; Perfusion set (15 cm, ID 1.6 mm): Cat No. 10962; μ -slide^{0.4} Luer: Cat. No. 80176. ibidi GmbH, Munich, Germany) is well-suited for the cultivation of cells under flow conditions to simulate blood vessels. In this experiment, a continuous flow was generated. The pump system was constructed according to the instructions (IBIDI GmbH, AN 13: HUVECs under Perfusion). 350,000 primary HUVEC cells were seeded onto a μ -slide I^{0.4} Luer and incubated for 24 h at 37 °C and 5% CO₂ in the incubator to form a monolayer. The next day, the ibidi flow application starts. The experiment was conducted under the following conditions: 31.9 mbar pressure, 21.70 ml/min flow rate, viscosity 0.007 (dynes)/cm² and a shear stress of 20 dyn/cm². Static conditions were also tested on the same ibidi μ -slides^{0.6} without shear application.

The cells in MS1 culture are placed in 6-well plates (n = 3) at a concentration of 200,000 cells/ml for each well. The cells were then placed at 37 °C as control and the cells under flow were placed under agitation of our laboratory digital orbital shaker for 72 hours or 24 hours at 210 rpm, either a speed corresponding to 12 dynes / cm² (Salek, Sattari, and Martinuzzi 2012) or to 260rpm.

11. Migration Assay

For the migration assay, *ibidi*TM culture inserts 2-well in μ -well (cat# 81176, Germany) were used. Briefly, the inserts consist of two chambers separated by a 0.5mm divider, each chamber with a growth area of 0.22cm². The inserts were planted into 35mm tissue culture dishes using sterile tweezers. After HUVECs were transfected using the previously described protocol, cell suspensions were prepared at 7-9x10⁵ cells/ml in medium, of which 70 μ l was transferred to each chamber. The cells were left to adhere and grow to ~100% confluence.

The dividing strip between the chambers was removed and images were acquired along the cell-free zone between time 0 and 8 hours. Cell migration was assessed using ImageJ software, measuring the area covered by the cells. The encroachment of the cell-free gap for each transfected cell type was determined by comparing results to the 0h time point.

12. Intracellular Calcium Measurement

Three days after HUVECs transfection, cells in 96-well plate were incubated for one hour at room temperature with reduced light with Fura-2-acetoxymethyl ester (Fura-2 AM, 2 μ M) in HBSS buffer (CaCl₂ 2.5mM, MgCl₂ 1mM, HEPES 5mM and BSA 0.5%, pH=7.3). Cells were washed twice, and then incubated for one hour with HBSS buffer before fluorescence measurement. The fluorescence measurements were performed with a Flex Station 3 from Molecular Devices (Silicon Valley, USA). The inducers of different concentrations were prepared in a separated plate and added robotically with a liquid handler by the Flex

Station. The fluorescence emitted by Fura-2 AM at 510 nm was measured upon excitation at 340 nm (Ca²⁺ bound Fura-2) and 380 nm (Ca²⁺ free Fura-2). The ratio of the fluorescence signals and the resulting AUC (area under the curve) were then calculated with GraphPad Prism (GraphPad Software, San Diego, USA).

13. Analysis of mRNA levels by qRT-PCR in cells

The RNAs of cells subjected to oxidative stress and those of cells under flow are then extracted using an RNeasy Lipid Tissue Mini Kitmini (Qiagen - ref 74804). The cells are solubilized in an RLT + β -mercapto-ethanol lysis buffer. Several washing steps and an elution step are carried out on a column according to the manufacturer's recommendations. The RNAs are then stored after extraction at -20 °C until use. After spectrophotometric determination of the quantity and quality of RNA present in each sample (NanoDrop 2000, ThermoFisher Scientific), an qRT-PCR is carried out. The reverse transcription is performed using a QIAGEN quantitect® kit. The reverse transcription program contains a cycle of 30 min at 42 °C and 3 min at 95 °C allowing the dissociation of the enzyme. The cDNAs obtained are stored at -20 °C and the PCR is carried out using an intercalating agent: SybrGreen (SYBR® Select Master Mix - ThermoFisher Scientific). The cDNAs are diluted 1:50 and placed in a 96-well plate containing, per well, 6 μ l of mix of oligonucleotides (5 μ M) + SybrGreen and 4 μ l (corresponding to 10 ng) of sample are deposited. The amplification program and the plate reading are performed using the LightCycler® 480 (Roche). The program contains an activation cycle (95 °C for 3 minutes), 40 amplification cycles (95 °C for 15 seconds, then 60 °C for 1 minute) then a dissociation curve.

Gene Name	Nucleotide Sequence (5'-3') Forward (F) and Reverse (R)	Gene Name	Nucleotide Sequence (5'-3') Forward (F) and Reverse (R)
<i>Klf2</i>	F-acagactgctattattggaccttag R-cagaactggtggcagagtcatt	<i>Dnm1l</i>	F-agatcgtcgtagtggggaacg R-ccactaggctttccagcact
<i>Nos3</i>	F-ccagtgccctgcttcac R-gcagggcaagtaggatcag	<i>Mfn1</i>	F-aaaacatactggactcagtaaacgtg R-ggtcttccctctctccattg
<i>Cyp1b1</i>	F-agccaggacaccctttcc R-cctgaacatccgggtatctg	<i>Mfn2</i>	F-cattctgtggtcggaggag R-aaggagagggcgatgagtct
<i>Edn1</i>	F-tgctgtcgtgactttccaa R-gggtctgcactccattct	<i>Fis1</i>	F-gagaccttagtggaggcttca R-tcagactgaaattccttcaaaat
<i>Thbd</i>	F-tggcgataaccacaccagt R-agagtggagccgtctggtc	<i>Casp3</i>	F-tggaggctgactctctgtatg R-cacgaccgtccttgaa
<i>Entpd1</i>	F-ctctgcaaggtataactcac R-gcgttgctgtctttgatcttg	<i>Casp 7</i>	F-ccgtccacaatgactgctc R-ccgagttgctgtgtctct
<i>Pecam1</i>	F-cggtgttcagcgagatcc R-cgacaggatggaaatcacia	<i>Nox4</i>	F-ctggaagaaccaagtcca R-aaaggcacaagggtccagaa
<i>Ace</i>	F-ggaacaagtcgatgtagagaagc R-acagaggtacactgctgatct	<i>Hprt</i>	F-tgatagatccattctatgactgtaga R-aagacattcttccagttaaagttgag
<i>Opal</i>	F-accaggagaagtagactgtgcaaa R-tcttcaataaacgcagaggtg	<i>Gusb</i>	F-ctctggtggccttacctgat R-cagttgtgtcaccctcacctc
<i>Gapdh</i>	F-ccggggctggcattgctctc R-ggggtgggtggtccagggtt	<i>OPA1</i>	F-ccgctagaagggtgaagt R-gcgaggctggtagccatatt

<i>DNMIL</i>	F-ccaatgggtgccggctt R-tgtgaagacgtctggagc	<i>GAPDH</i>	F-gacagtcagccgcatcttct R-gcgccaatacgaacaaatc
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Table 2 List of primers used for PCR experiments

Statistical analyses

The results were expressed mean \pm SEM. The significance of differences between the results is determined between groups by the analysis of the variance using the Two-way ANOVA for repeated measures. When an interaction was observed between the 2 factors, the effect of OPA1 and DRP1 deficiency was studied in each genotype using a Bonferroni post-test. To test gene deficiency in the two groups of mice, Kruskal-Wallis and Mann-Whitney tests were used. A value of $P < 0.05$ was considered as statistically significant. All photos and tables were analyzed using by the GraphPad Prism 6.0.

Results

1. Introduction

All the vascular system is aligned with endothelial cells from the internal side in contact with blood flow. But, several factors affect blood flow including factors like blood pressure, fluidic viscosity, arterial flow resistance, and neurosynaptic perturbations. As blood flow is in contact with the endothelium, wherever blood flow acts, shear stress exists. Shear stress is defined by the force per unit area created when a tangential force (blood flow) acts on a surface which is in the case of arteries, defined as the endothelium. Blood flow regulates vascular tone and structure through the mechanotransduction of mechanical forces into biochemical intracellular signals that acts in different pathways to maintain vascular homeostasis. This homeostasis is maintained through the action of different vasoactive molecules including NO, PGI₂ and EDHF, which act on the vSMCs to change vascular tone. In addition, shear stress can directly act on different pathways that acts directly on endothelial genome to alter gene expressions, change cell morphology and metabolism.

The vascular endothelium has a central role in vascular homeostasis and an endothelial dysfunction is the hallmark of all cardiovascular disorders (Struijker-Boudier 2007). Changes in mitochondrial bioenergetics have been long described in most metabolic, neuro- and cardio-vascular disorders and it is now well admitted that alteration in mitochondrial bioenergetics contributes to these diseases (Murphy 2016). Indeed, the alteration in mitochondrial bioenergetics has been evidenced in tissues with high-energy demand such as the heart (Tilokani et al. 2018) where the involvement of mitochondrial dynamics in these disorders has only recently been shown (L. Chen et al. 2009). Indeed, mitochondria are highly dynamic structures, altering their inner and outer membranes by means of fission and fusion mechanisms. The fission process is mediated by the cytosolic dynamin proteins DRP1 and DNM2, and the outer-membrane human fission factors Fis1, MFF, MID49 and MID51. On the other hand, mitochondrial fusion is mediated by the outer-membrane Mitofusins 1 & 2 and the inner-membrane optic atrophy factor 1 (OPA1) (Malka, Lombès, and Rojo 2006). Pharmacological inhibition of mitochondrial fission has protective activity in heart ischemia-reperfusion injury (Ong et al. 2010a). In pulmonary artery smooth muscle cells (SMCs) from hypertensive mice; hyper-proliferation is associated with mitochondrial fragmentation (Marsboom et al. 2012). Similarly, mitochondrial fission in SMCs is essential for PDGF-induced proliferation (Salabei and Hill 2013).

A role for mitochondrial dynamics in endothelial cells (ECs) functions has recently been shown. Indeed, ECs are sensitive to oxidative stress of mitochondrial origin, and ECs mitochondria undergo hyper-fragmentation in cardiovascular diseases (Marín-García 2016). Although, vascular ECs do not depend on mitochondria for their energy demands, as they mainly rely on glycolysis to produce ATP (Culic, Gruwel, and Schrader 1997), it is hypothesized that ECs mitochondria play a regulatory rather than an energy-

producing role (Kadlec et al. 2016). The pathway regulated by mitochondrial dynamics in the endothelium remains to be uncovered. Interestingly, fusion and fission are sensitive to shear stress in cultured ECs (HUVECs, BAECs) (Bretón-Romero et al. 2014) and shear stress-dependent Ca^{2+} mobilization in HUVECs relies on mitochondrial-dependent activations of endoplasmic reticulum channels (Scheitlin et al., n.d.), indicating that the onset of a biological regulation related to mitochondrial dynamics is prompt, without needing the transcription and translation processes of proteins related to mitochondria. Although these studies strongly suggest a role of the mitochondria in ECs function, the role of the fusion and fission remains unclear. Furthermore, the role of mitochondria dynamics on ECs-dependent dilatation and in endothelium dysfunction-associated disorders is unknown.

As endothelial dysfunction, evidenced by reduced flow-mediated dilatation (FMD) occurs very early in most cardiovascular diseases (Struijker-Boudier 2007), we hypothesize that mitochondrial dynamics could play a role in the onset of cardiovascular disorders and in their time-course. The present study aimed at deciphering the role of mitochondrial dynamics in EC homeostasis and in atherosclerosis, which occurs in area of disturbed flow (shear stress) leading to endothelial dysfunction.

2. Role of OPA1 in the vascular reactivity

In order to study the effect of mitochondrial fusion on the response to flow, we have isolated mesenteric arteries from two strains *Opa1*^{+/+} WT and *Opa1*^{+/-} mice, and analyzed the flow-mediated dilation (FMD) by arteriography in vitro (experiments performed by C. Proux, Engineer in Mitovasc).

Our results have revealed that at high flow levels of 30 to 50 μ L/min, FMD was about 50% lower in male *Opa1*^{+/-} compared to male *Opa1*^{+/+} mice. However, there was no significant difference in arterial diameter between *Opa1*^{+/-} and *Opa1*^{+/+} male mice, indicating that the structure of the vessels between the two strains is probably not changed. OPA1 is possibly affecting FMD only as Ach-mediated dilation, and KCl (80 mM)-mediated contraction were not affected Figure 36.

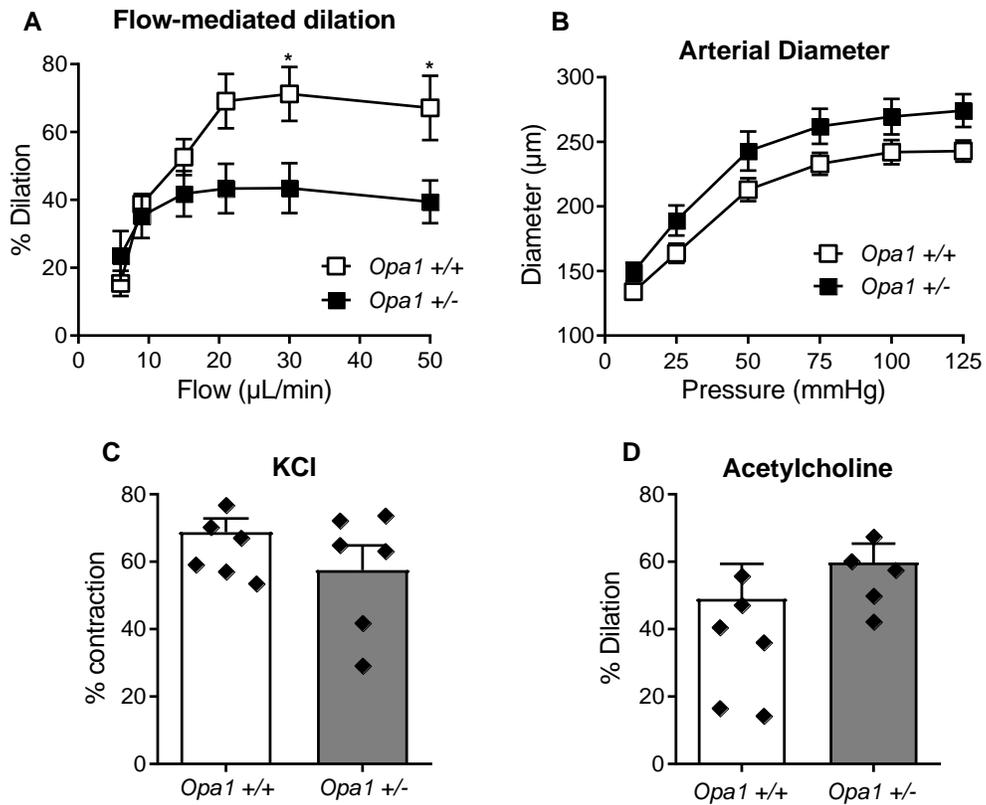


Figure 36 Vascular reactivity in mesenteric arteries of *Opa1*^{+/-} male mice using arteriography, flow-mediated dilation (A) arterial diameter in response to the pressure steps (B), KCl (80mM)-mediated contraction (C) and Ach (1µM)-mediated dilation. Values are expressed as mean ± SEM with 6 mice in average per group. **P*<0.05 *Opa1*^{+/-} compared to *Opa1*^{+/+}. Two-way ANOVA for repeated measurements (A,B); and Mann-Whitney test (C, D).

Female *Opa1*^{+/-} mice had a significant decrease in FMD compared to *Opa1*^{+/+} mice, without significant difference in the passive diameter, Ach-mediated dilation and KCl-mediated contraction (Figure 37).

These results suggest that OPA1 plays an important and possibly selective role in FMD.

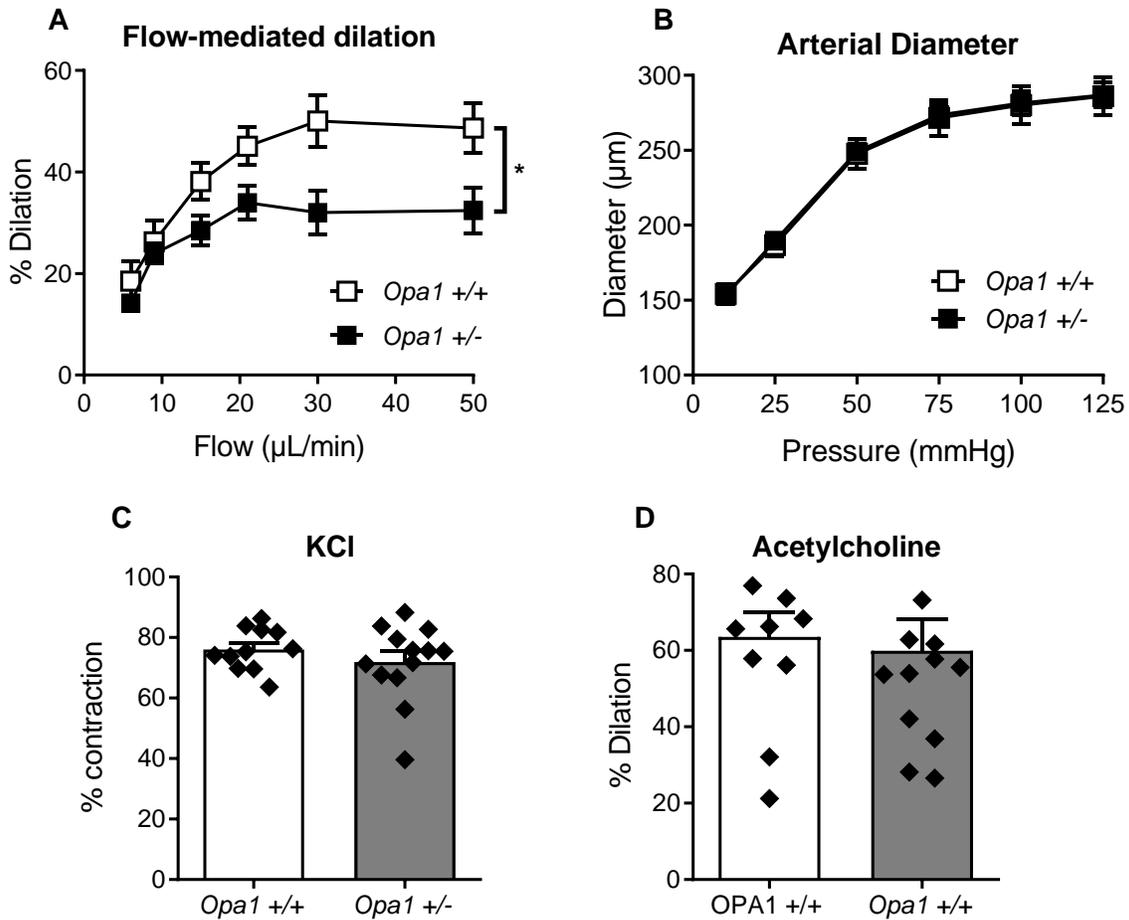


Figure 37 Vascular reactivity in mesenteric arteries of *Opa1*^{+/-} female mice using arteriography, flow-mediated dilation (A) arterial diameter in response to the pressure steps (B), KCl (80mM)-mediated contraction (C) and Ach (1µM)-mediated dilation Values are expressed as mean ± SEM with 11 mice in average per group. *P<0.05 *Opa1*^{+/-} compared to *Opa1*^{+/+}. two-way ANOVA for repeated measurements (A, B); and Mann-Whitney test (C, D).

Moreover, we have studied the effect of *Opa1* gene knocking out on the response to flow in female ovariectomized mice. These results were not enough to obtain a clear conclusion from these experiments, thus further experiments will be done to correlate between the female estrogens and OPA1 functioning in the vascular reactivity.

3. Role of mitochondrial dynamics in endothelial functioning

Endothelial cells are highly affected by mitochondrial metabolism, as the mitochondria in ECs functions more in cell signaling than a powerhouse of the cell. But, the mitochondrial signaling role is highly affected by the balance in mitochondrial dynamics. Thus, we hypothesized that the balance in mitochondrial dynamics highly affects mitochondrial functioning and vice-versa.

3.1. Role of mitochondrial dynamics on calcium signaling in ECs

As calcium is a main second messenger for the activation of the dilatory machinery in ECs, leading to activation of different signaling pathways that allows the release of NO, EDHF, and AA, we measured the intracellular levels of calcium Ca^{2+}_i in ECs. Different agonists can stimulate EC surface receptors to induce calcium signaling (Ach, Histamine, Bradykinin, Insulin, ATP, 17 β -estradiol and many others). So, in our project, we have stimulated HUVECs with ATP and Histamine to induce calcium signaling.

To examine whether the effects of OPA1 on flow-dependent dilation was endothelium dependent or not, the measurement of the intracellular calcium levels released after stimulation of cells lacking *OPA1* was performed.

HUVECs were transfected with *siOPA1* and *siDNM1L* that silences the genes responsible for OPA1 and DRP1 expression, respectively. After 72 hours of transfection, the time for the loss of the total OPA1 and DRP1, these cells were loaded with FURA-2 and stimulated with ATP or Histamine. In HUVECs transfected with *siDnm1l*, ATP-mediated increase in Ca^{2+}_i was not different from *siCtl* (only with 0.1 μ M ATP). In contrast, cells with *siOPA1* and *siDNM1L* have shown a significant decrease compared to *siCTL* when stimulated with histamine (Figure 38).

These results indicated that mitochondrial fission and fusion directly affects the calcium signaling response to external factors. In addition, this difference in the intracellular calcium liberated after stimulation can lead to the reduced vascular reactivity in flow mediated dilation.

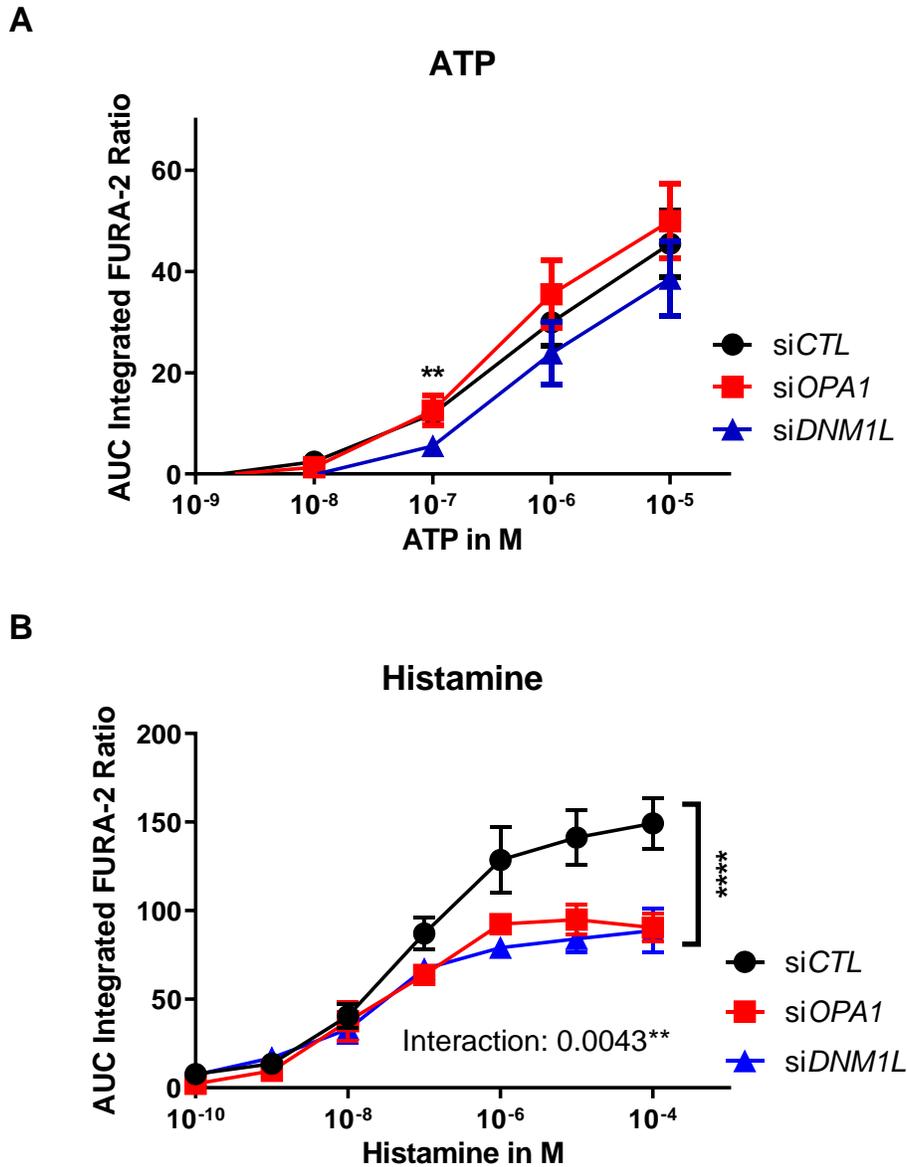


Figure 38 Increase in intracytosolic Calcium (Ca^{2+}_i) induced by increasing concentrations of (A) ATP, and (B) Histamine in HUVECs. area under the curve of integrated FURA-2 ratio of the two wave lengths emitted, in each single concentration use are represented **** $P < 0.0001$ between different genes. Statistical test used is Two-way ANOVA. $N=3$ different experiments were performed.

3.2. Role of mitochondrial dynamics on ECs migration

Because cell migration is one of the main functions of endothelial cells, we checked whether the alteration in mitochondrial dynamics of ECs can alter their migration capacity. Transfected HUVECs were seeded after 72 hours from silencing in the Ibidi migration slides, where these cells were left for 2 hours as a period for attachment of the cells (starting period) (Figure 39A). Then, images were taken after 2, 4, 6, and 8 hours

from the starting period (Figure 39B). The obtained images were analyzed by calculating the area covered by the cells compared to the starting area after the cells were attached. We observed a significant reduction in the capability of migration of the HUVECs with siOPA1 compared to siCTL and siDNM1L cells, whereas siDNM1L cells had a migration capability similar to that of siCTL cells (Figure 39C). Taken together, our result suggest that mitochondrial dynamics plays a major role in endothelial functioning, where the decrease in the balance of mitochondrial fusion can lead to endothelial dysfunction.

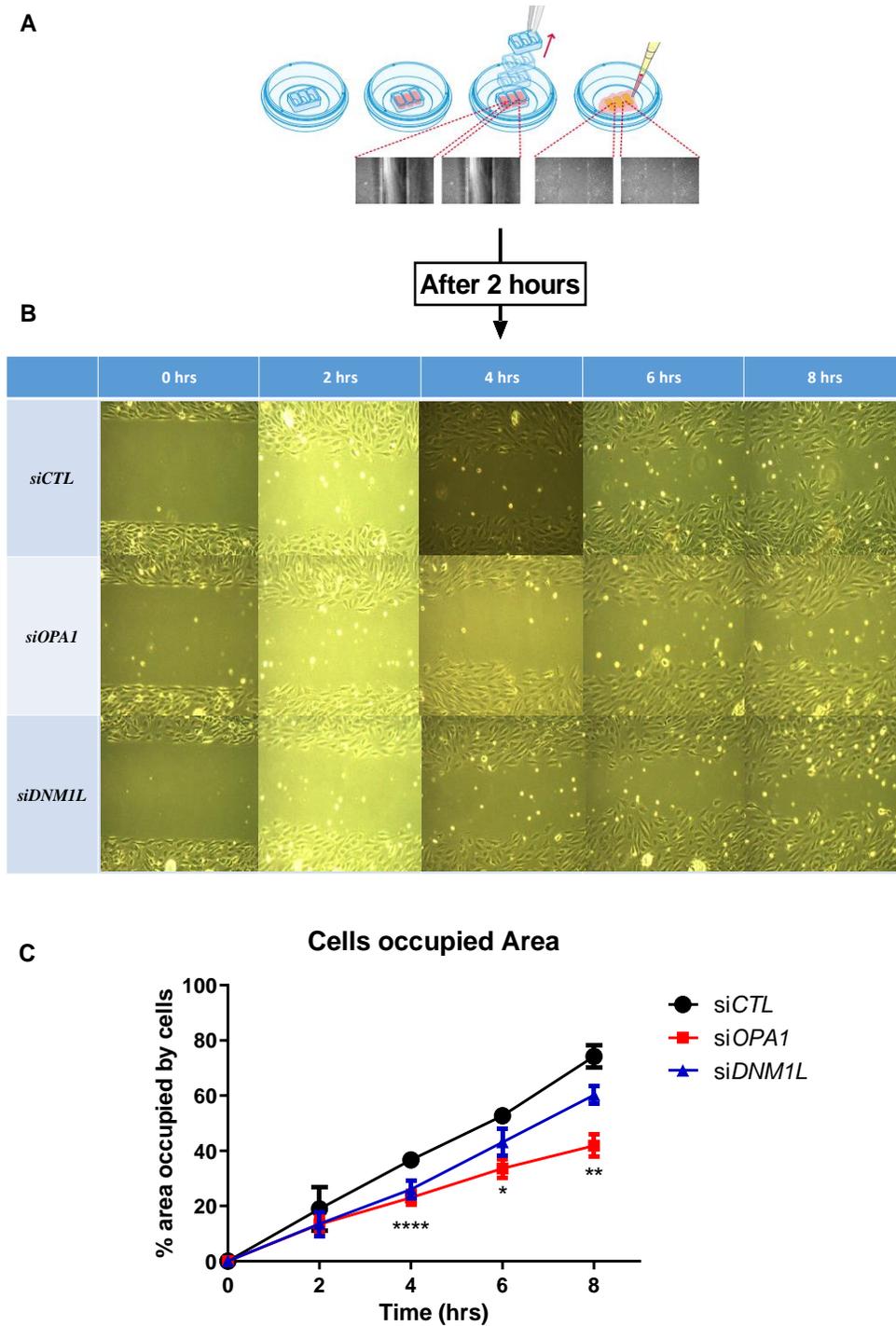
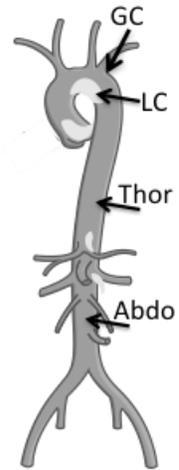


Figure 39 Endothelial cells migration in the absence of mitochondrial dynamic proteins. Cells were transfected for 72 hours and then seeded in (A) Ibidi μ -slides 0.4 and kept until attachment for 2 hours, where cells are allowed to migrate and (B) imaged at different time points using inverted microscope equipped with a camera. Then, (C) images were analyzed depending on the area occupied by the cells after each time point. * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$ of *siOPA1* compared to *siCTL*. Three independent experiments were done.

4. Mitochondrial Dynamics in different aortic zones

The aorta is the largest artery in the body that originates from the left ventricle of the heart and continues to the abdomen where it splits into common iliac arteries. Anatomically, different zones of the aorta are subjected to different types of shear stress. The anatomy of the aorta plays a major role in identifying the pattern of shear stress in the aorta. Therefore, the aorta is divided into sections of ascending aorta (AAo greater curvature, GC), aortic arch (AA/lower curvature LC), descending aorta or thoracic aorta (ThAo), and abdominal aorta (AbAo) (see the schema next). In general, in the zones with low or no curvature, blood flow generates laminar shear stress LSS, whereas in the zones of aortic curvatures or branching points of the aorta, the blood flow generates a disturbed flow.



To better understand the effect of flow on mitochondrial dynamics, male *Ldlr*^{-/-} mice were fed with standard diet for 6 months and then the aorta was dissected and separated in four parts depending on the different zones of shear stress. We have studied the mRNA relative expression of mitochondrial dynamic proteins. *Opa1*, *Mfn1*, *Mfn2*, *Dnm1l* and *Fis1* mRNA expression levels were assessed in the four different parts of the aorta (Figure 40). This was done by Jade Auriere during her Master 2 training period.

We found that in the zone of disturbed flow (LC), *Opa1* mRNA expression level was lower compared to other aortic zones. On the other hand, the mRNA expression levels of *Dnm1l* was approximately two times higher in the AA compared to the 3 other aortic zones. As a result, disturbed flow, low or oscillatory, decreases the events of mitochondrial fusion and favors mitochondrial fission.

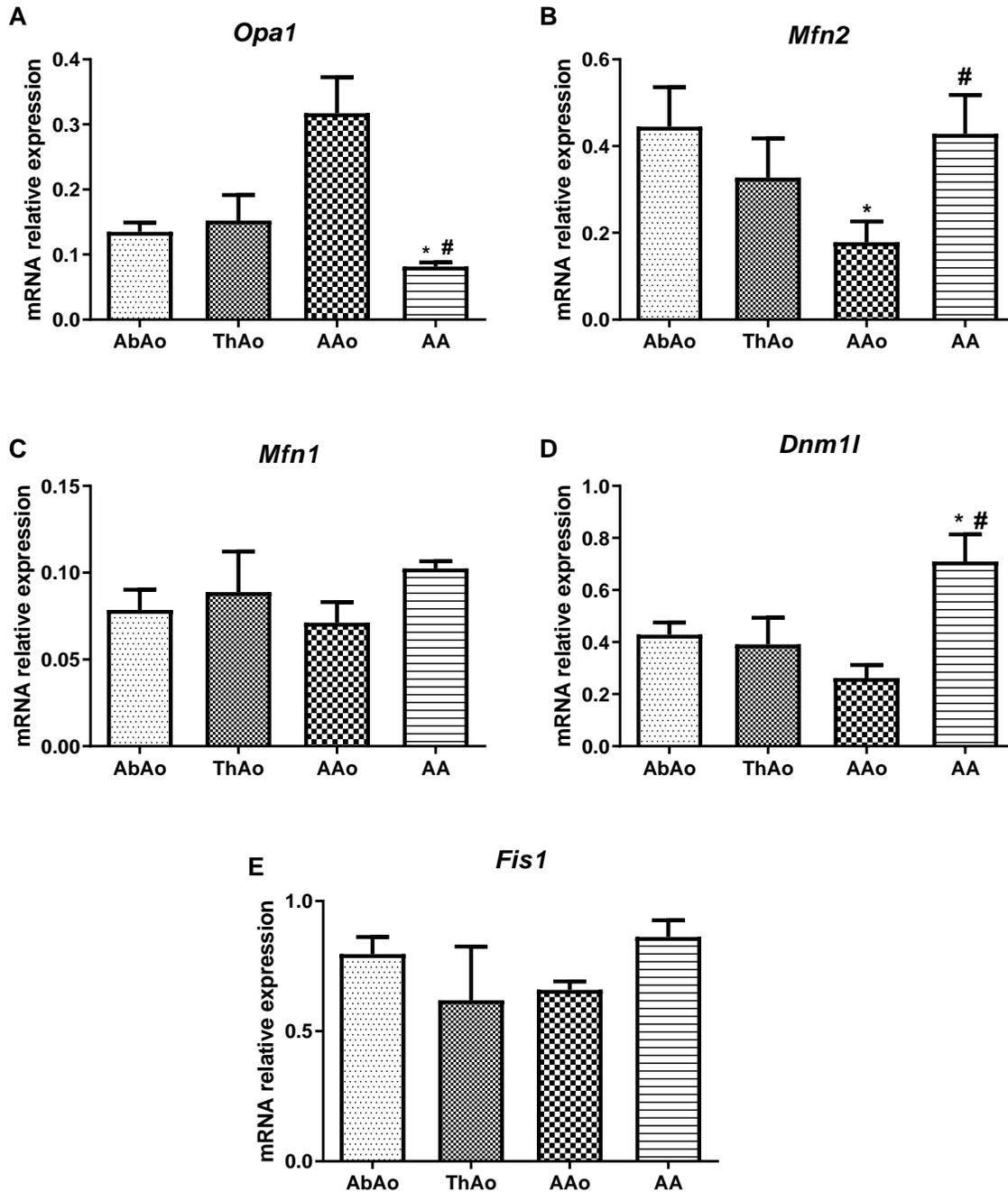


Figure 40 Mitochondrial dynamics mRNA expression levels in different aortic zones. Values are expressed as mean \pm SEM with 6 mice per group. * $P < 0.05$ compared to ThAo and AbAo; # $p < 0.05$ compared to AAo. Statistical test used is Mann-Whitney test.

On the other hand, *Ldlr*^{-/-} mice males fed with HFD for 4 months have shown a significant decrease in the mRNA levels of *Opa1*, *Mfn1*, *Mfn2* and *Fis1* of the AA compared to other aortic zones (Figure 41). Thus, these results reveal the dysregulation in the mitochondrial dynamics of the zones subjected to a

disturbed flow. As these zones are the most susceptible zoned to atherosclerotic lesions by the disturbed flow, we can conclude that the disturbed flow destabilizes mitochondrial dynamics and confirms its importance in the formation of atherosclerotic lipid deposition.

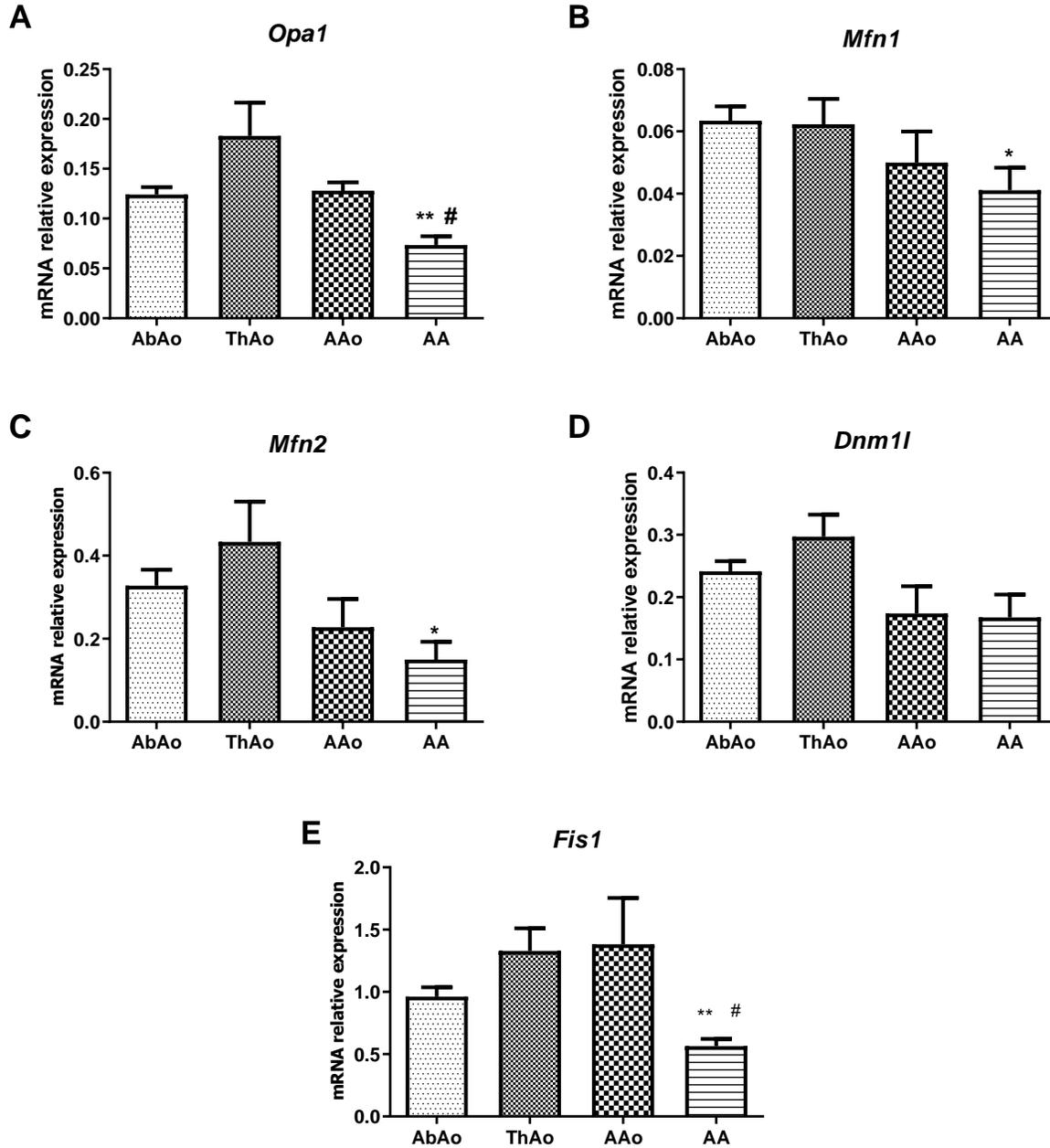


Figure 41 Mitochondrial dynamics in different aortic zones in mice fed with high fat diet. Values are expressed as mean \pm SEM with 6 mice per group. * $P < 0.05$ AA in comparison to ThAo and AbAo; ** $P < 0.01$ AA in comparison to AAo; # $P < 0.05$ in comparison to AA. Statistical test used was Mann-Whitney test.

Moreover, in order to check if a variation in the flow level can affect the regulation of mitochondrial dynamics in small resistant arteries, we have used a model of flow mediated outward remodeling. In this model, the mesenteric arteries were ligated for one week as shown in Figure 42, to generate three different level of flow including normal flow NF, high flow HF and low flow LF. Noting that, in the 3 conditions, flow is laminar.

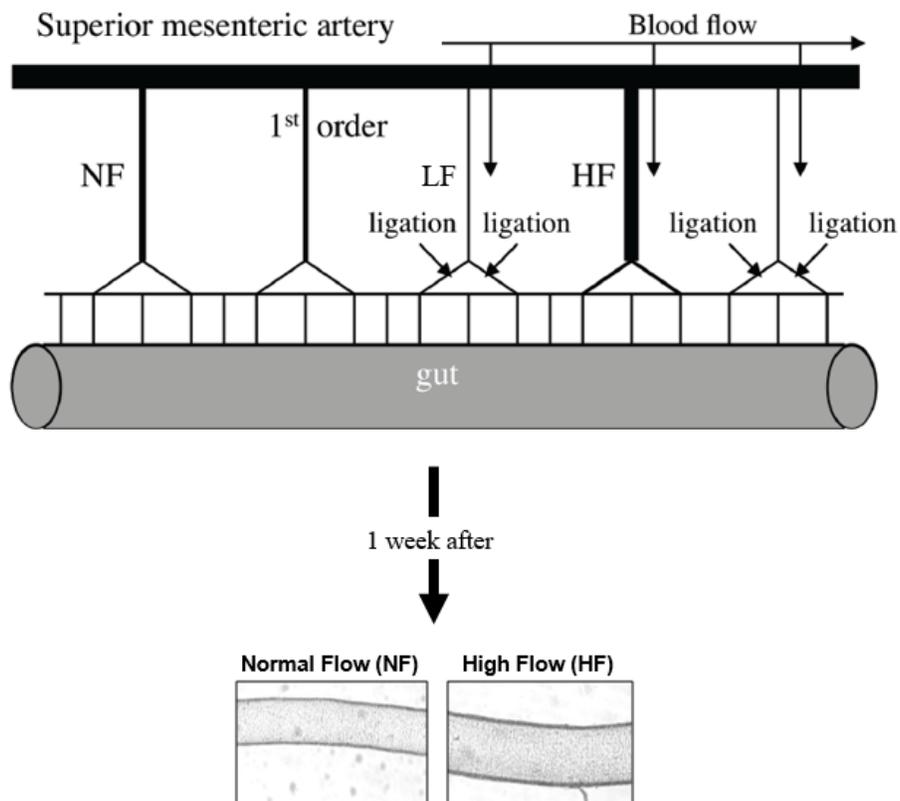


Figure 42 Flow-mediated outward remodeling of mesenteric arteries. Arteries were ligated in the zones marked by ligation to generate arteries with three types of flow, NF: Normal Flow, HF: High Flow, and LF: Low Flow. (Belin de Chantemèle et al. 2009)

Then, we have assessed the levels of mRNA of the mitochondrial dynamics proteins. This protocol was performed by A.L. Guihot (Engineer) and Pauline Robert (Ph.D. student).

qRT-PCR analysis results revealed that there was no difference in mRNA expression levels of *Opa1*, *Dnm11*, *Mfn1* and *Mfn2* in the arteries with the three different types of flow (Figure 43 A, B, C, D). However, the mRNA expression level of *Nos3*, responsible for eNOS expression, was reduced in the arteries subjected to low flow compared to NF and HF arteries (Figure 43 E). In contrast, these LF arteries have shown an increase in mRNA expression levels of *Edn1*, the gene responsible for ET-1 expression, compared to NF and HF arteries (Figure 43 F). As a result, the change in laminar flow in small resistant arteries in vivo did

not affect the regulation of mitochondrial dynamics but it alters the response of endothelial cells to flow through the change in the levels of vasoconstrictors and vasodilators.

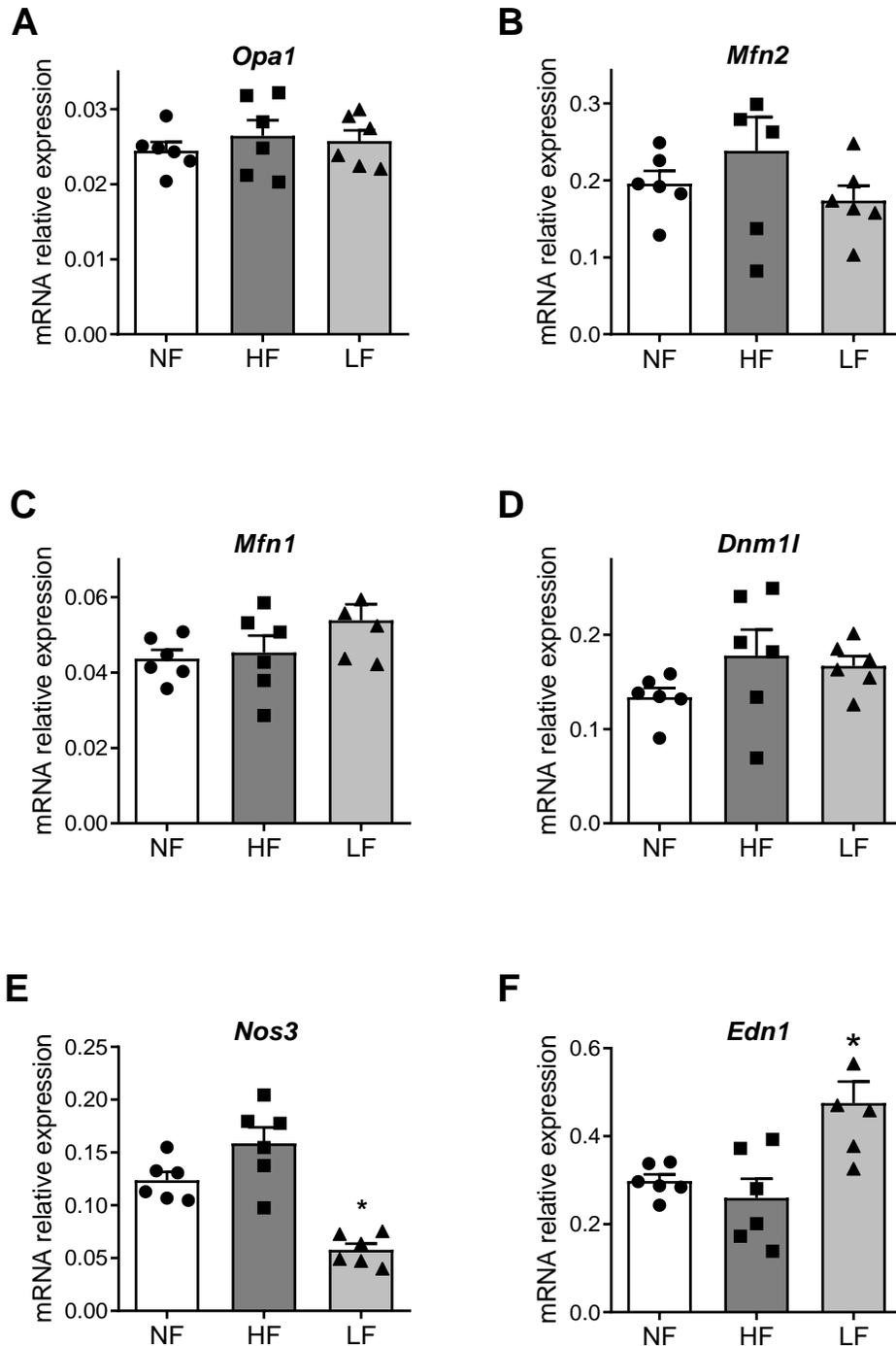


Figure 43 The effect of flow on the mRNA expression of different proteins in first order mesenteric arteries after flow mediated outward remodeling. (A, B and C) The changes in mitochondrial fusion proteins mRNA relative expressions in the three different types of arteries. (D) The change in mitochondrial fission protein DRP1 mRNA

relative expression in the different types of arteries with three different flow velocities. (E) The relative mRNA expression of eNOS protein in the three arterial types. (F) The changes in mRNA relative expression of ET-1 protein. NF: Normal Flow, HF: High Flow, LF: Low Flow. * $P < 0.05$ NF versus LF with statistical test of Kruskal Wallis test.

5. Role of Mitochondrial Dynamics in Atherosclerosis

5.1. OPA1 reduces the formation of lipid deposit in mice

Since normal mice cannot develop atherosclerotic plaque in their arteries, we have tested if the mice lacking Opa1 gene can develop atherosclerotic plaque or not. *Opa1*^{+/-} mice haven't shown any lipid deposit in their aortas compared to WT *Opa1*^{+/+} mice whether they were fed a high-fat diet or not. Thus, we have generated a new mouse model by crossing the *Apo1*^{+/-} mice with *Ldlr*^{-/-} mice which are known to develop lipid deposits when fed a high-fat diet.

Male *Ldlr*^{-/-} *Opa1*^{+/-} mice fed a HFD

In order to check for the difference in the lipid deposit formation in male mice lacking OPA1 protein, we have used male mice with a double KO: *Ldlr*^{-/-} *Opa1*^{+/+} and *Ldlr*^{-/-} *Opa1*^{+/-} mice. After feeding these mice a high fat diet (HFD) for 4 months starting at the age of 2 months. Both strains show lipid deposits on their aorta. Lipid deposits were significantly larger in male *Ldlr*^{-/-} *Opa1*^{+/-} mice compared to male *Ldlr*^{-/-} *Opa1*^{+/+} mice (Figure 44 A). Thus, in male mice, OPA1 could play a protective role during the development of atherosclerotic plaque. Although these mice have developed more lipid deposits than WT mice, their weight, glycaemia, perigonadic fat, and liver weight haven't shown any significant difference (Figure 44 B). Experiment was done by Mrs. Anne-Laure Guihot, an engineer in the MitoVasc laboratory.

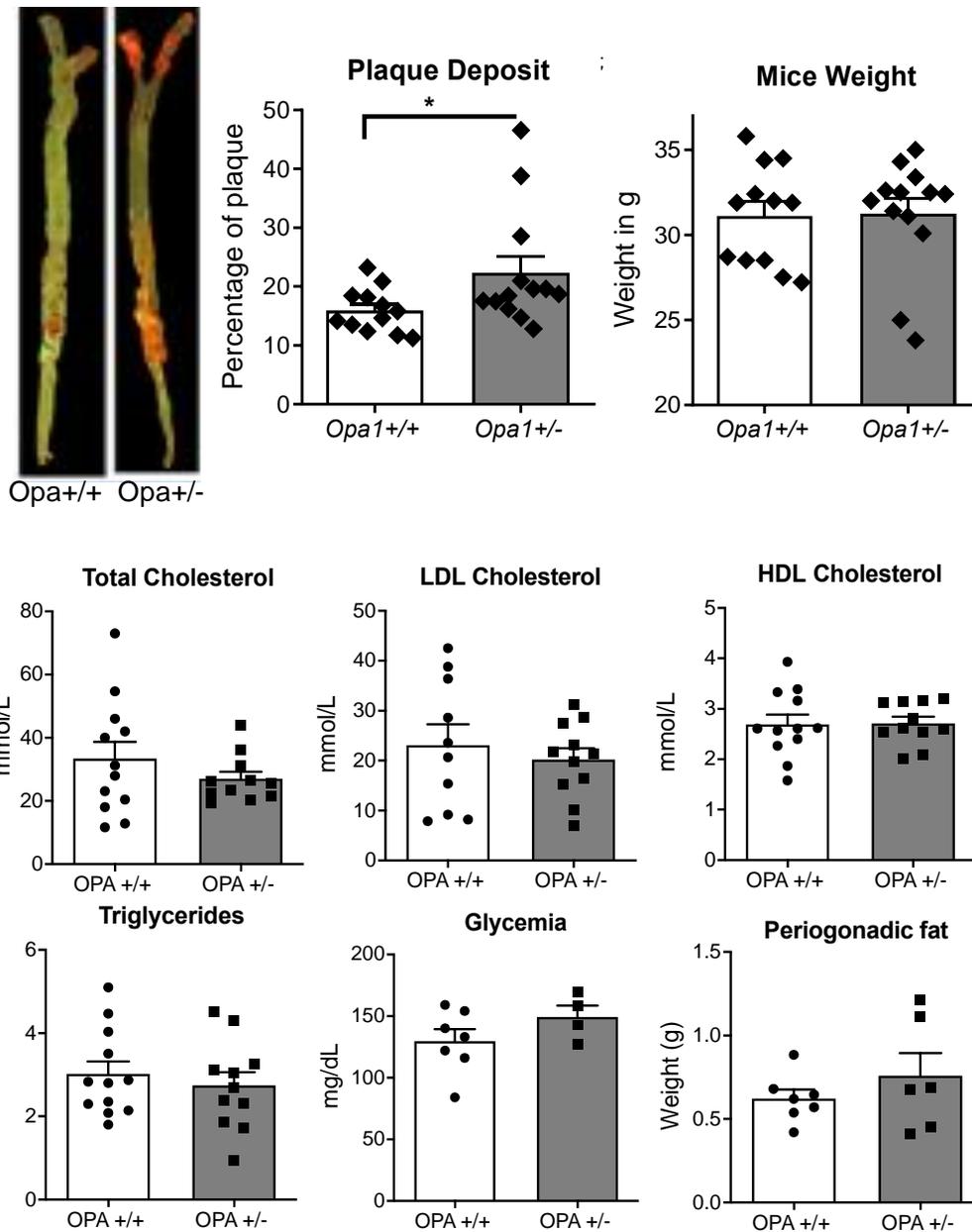


Figure 44 Male *Ldlr*^{-/-} *Opa1*^{+/+} and *Ldlr*^{-/-} *Opa1*^{+/-} mice aortic plaque formation analysis using (A) Oil red O test coloration. (B) This plaque was quantified as the percentage of plaque formed in the two mice strains. (C, D, and E) represents the different metabolic parameters including mice weight, glycemia and liver weight. 13 mice from each group were used. **P*<0.05, Mann-Whitney test was performed.

Female *Ldlr*^{-/-} *Opa1*^{+/-} mice fed with HFD

Women are better protected than men against cardiovascular diseases due, in part, to the estrogen actions. Indeed, women usually develop atherosclerosis 10 years later than men. Once women reach menopause, the risk of developing atherosclerosis increases due to the loss in the estrogen protective activity. To check whether estrogen activity in protection from atherosclerosis changes in mice lacking OPA1, we used female

Ldlr^{-/-} *Opa1*^{+/+} and *Ldlr*^{-/-} *Opa1*^{+/-} mice and fed them HFD for 4 months. We observed no significant difference in the lipid deposit between *Ldlr*^{-/-} *Opa1*^{+/+} and *Ldlr*^{-/-} *Opa1*^{+/-} mice (Figure 45A&B). Besides, these two strains haven't shown any significant difference in weight, glycaemia, liver weight and perigonadic fat (Figure 45 C, D, E&F). Mrs. Jade Auriere analyzed part of the animals during her master 2 training perios in our laboratory.

Figure 45 Female *Ldlr*^{-/-} *Opa1*^{+/+} and *Ldlr*^{-/-} *Opa1*^{+/-} mice aortic plaque formation analysis using (A) Oil red O test imaged by an inverted microscope branched to a camera. (B) This plaque was quantified as the percentage of plaque formed in the two mice strains. (C,D, E, and F) represents the different metabolic parameters including mice weight, glycemia, liver weight and perigonadic fat. 10 mice per group were analyzed. Mann-Whitney test was performed as the statistical test.

Ovariectomized female mice OPA1 protection

As the activity of estrogen retained OPA1 activity in atherosclerotic protection, *Ldlr*^{-/-} female mice were ovariectomized (OVX) and fed with HFD for 4 months. The amount of plaque formed in the aortas of these mice was quantified by Oil Red O test after aortas dissection. Female ovariectomization of mice lacking OPA1 have revealed the same results of male mice lacking this protein. That is, OVX *Ldlr*^{-/-} *Opa1*^{+/-} have shown an increase in the levels of atherosclerotic lipid plaque formed in the walls of the aorta compared to OVX *Ldlr*^{-/-} *Opa1*^{+/+} mice. Thus, these results confirm that the activity of estrogens in the compensation of the loss of OPA1, through their protective effect against plaque formation, is abolished by ovariectomization in females. Also, interestingly OVX *Ldlr*^{-/-} *Opa1*^{+/-} mice have significantly developed more fat around the gonads than OVX *Ldlr*^{-/-} *Opa1*^{+/+} characterized by perigonadic fat.

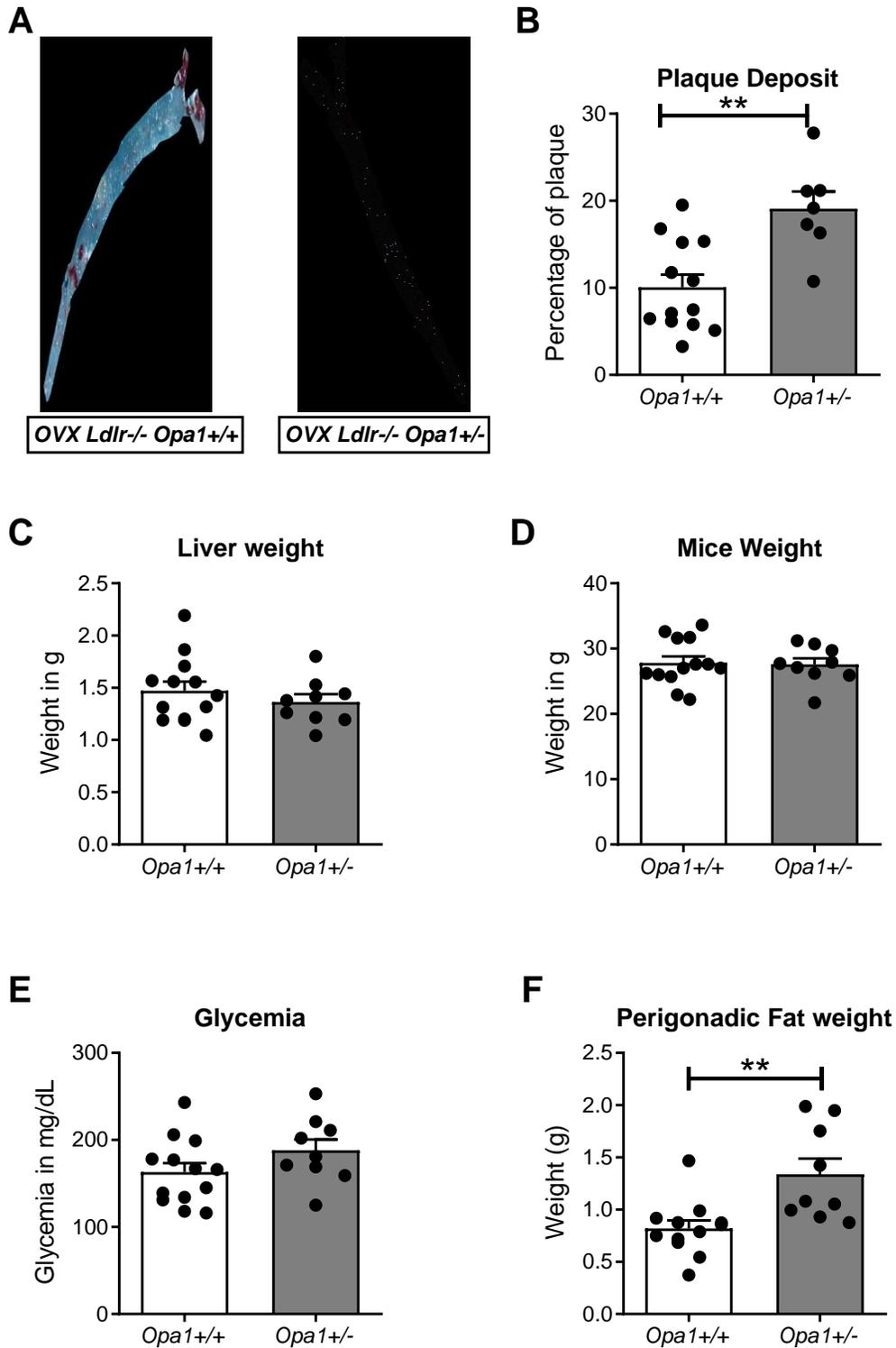


Figure 46 Females ovariectomized OVX Ldlr-/- Opa1+/+ and Ldlr-/- Opa1+/- mice aortic plaque formation analysis using (A) Oil red O test imaged by an inverted microscope branched to a camera. (B) This plaque was quantified as the percentage of plaque formed in the two mice strains. (C,D, E, and F) represents the different

metabolic parameters including mice weight, glycemia, liver weight and perigonadic fat percentage. 11 mice from each group were used. ** $P < 0.01$ with Mann-Whitney test performed as a statistical test.

5.2. DRP1 role in the enhancement of lipid deposit

Male *Ldlr*^{-/-} *Drp1*^{+/-} mice

In order to check for the difference in the lipid deposit formation in male mice lacking DRP1 protein, we have used male mice with the strains *Ldlr*^{-/-} *Drp1*^{+/+} as wild type, and *ldlr*^{-/-} *Drp1*^{+/-}. After feeding these mice with high fat diet HFD for 4 months starting from their 2 months old. Both strains were able to develop atherosclerotic plaque differently, where our results revealed a significant reduction in the levels of lipid plaque in *Ldlr*^{-/-} *Drp1*^{+/-} compared to *Ldlr*^{-/-} *Drp1*^{+/+} (Figure 47 A&B). Thus, in male mice, a reduction in the levels of DRP1 can play a protective role from the development of atherosclerotic plaque. However, the measurement of glycemic levels of these mice have shown a significant decrease in glycemic levels of *Ldlr*^{-/-}*Drp1*^{+/-} mice compared to *Ldlr*^{-/-}*Drp1*^{+/+} mice (Figure 47 C).

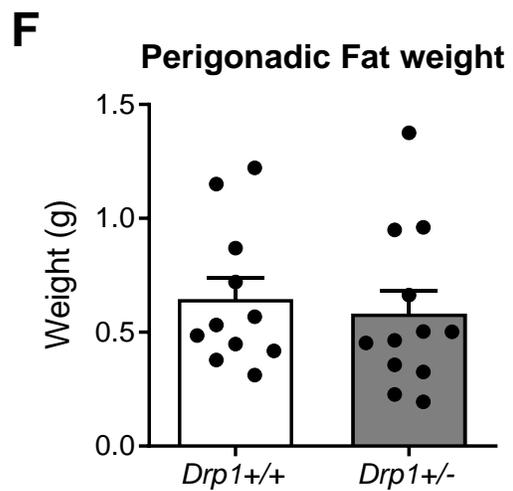
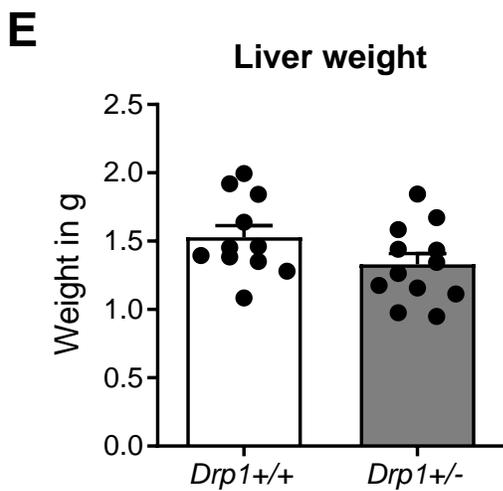
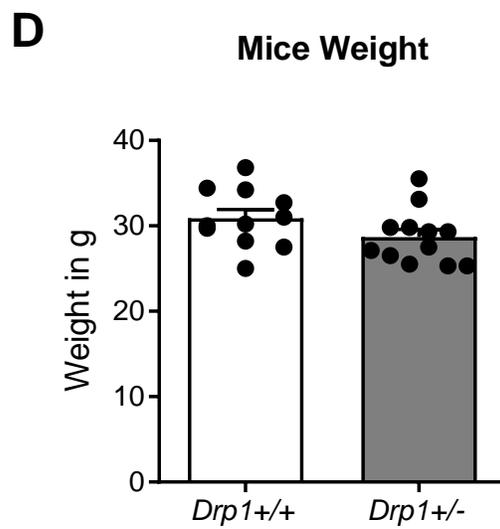
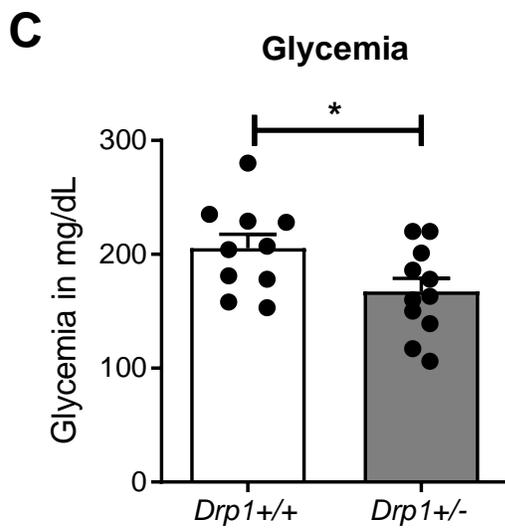
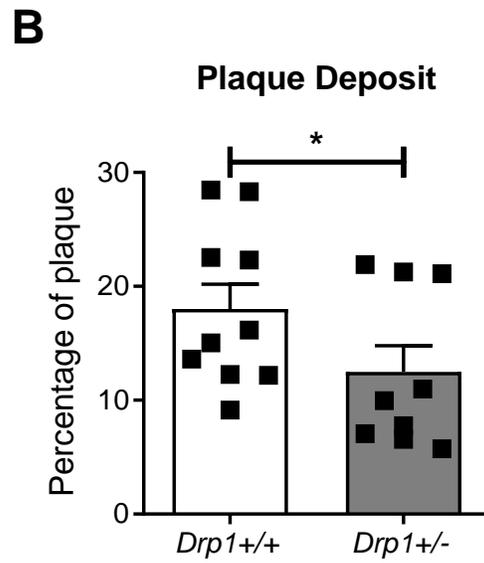
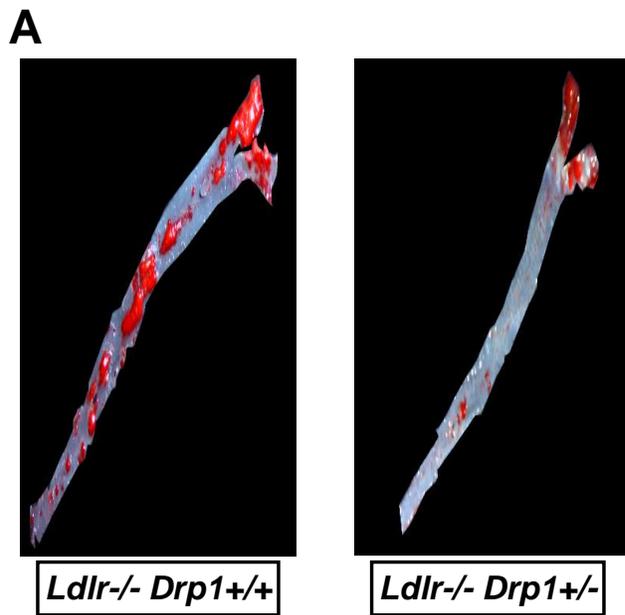


Figure 47 Male *Ldlr*^{-/-} *Drp1*^{+/+} and *Ldlr*^{-/-} *Drp1*^{+/-} mice aortic palque formation analysis using (A) Oil red O test imaged by an inverted microscope branched to a camera. (B) This plaque was quantified as the percentage of plaque formed in the two mice strains. (C,D, and E) represents the different metabolic parameters including mice weight, glycemia and liver weight. 12 mice from each group were used. Mann-Whitney test was used for statistical analysis with * $P < 0.05$ for plaque deposit and glycemia.

Female *Ldlr*^{-/-} *Drp1*^{+/-} mice

With the difference in the secretion of estrogens, female mice respond differently to shear stress. *Ldlr*^{-/-} *Drp1*^{+/+} and *Ldlr*^{-/-} *Drp1*^{+/-} mice were fed with HFD for 4 months and then, mice aortae were dissected. The amount of plaque deposit in the aortas were quantified using Oil Red O test in the two strains of mice. Interestingly, the protective effect of estrogen against atherosclerosis was abolished in the absence of DRP1, where *Ldlr*^{-/-} *Drp1*^{+/-} have shown about 40% significant increase in the plaque deposit compared to *Ldlr*^{-/-} *Drp1*^{+/+} mice (Figure 48 A&B). This change was not associated with any other change in other metabolic parameters including mice weight, Glycemia, liver weight, and perigonadic fat (Figure 48 C, D, E & F).

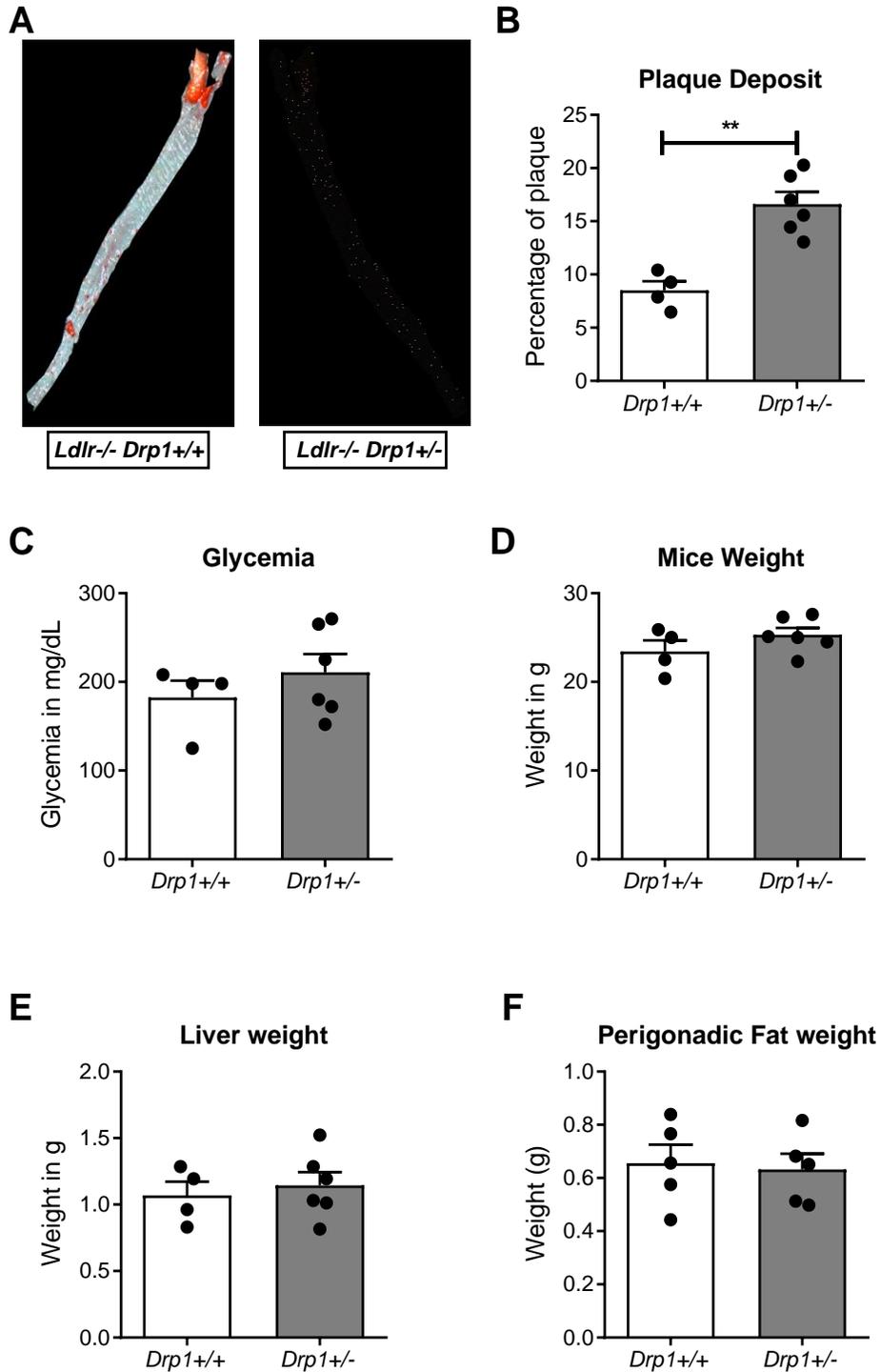


Figure 48 Female *Ldlr*^{-/-} *Drp1*^{+/+} and *Ldlr*^{-/-} *Drp1*^{+/-} mice aortic plaque formation analysis using (A) Oil red O test imaged by an inverted microscope branched to a camera. (B) This plaque was quantified as the percentage of plaque formed in the two mice strains. (C,D, and E) represents the different metabolic parameters including mice weight, glycemia and liver weight. 5 mice from each group were used. ***P*<0.001 using Mann-Whitney test for statistical significance test.

6. Endothelial cells response to flow

6.1. Endothelial cells alignment

As different zones of the aorta are subjected to different flow patterns and the endothelial cells are the first contact site between blood flow and the arterial wall, endothelial cells respond differently to the stress generated by flow. In order to study the impact of mitochondrial dynamics shear on the response of the endothelial cells to stress, we have started to apply laminar shear stress on HUVECs for 4 days. The duration of 4 days of shear application was chosen as the time needed for non-transfected HUVECs to align after shear exposure. HUVECs were transfected with siCTL, siOPA1 and siDNM1L for 72 hours and then seeded on ibidi slides where a flow of 20 dynes/cm² were applied to each slide at the same times. Images were taken during these four days where at day 4 (Figure 49), we have analyzed, using ImageJ, the percentage of the cells aligned from each of the transfected cells.

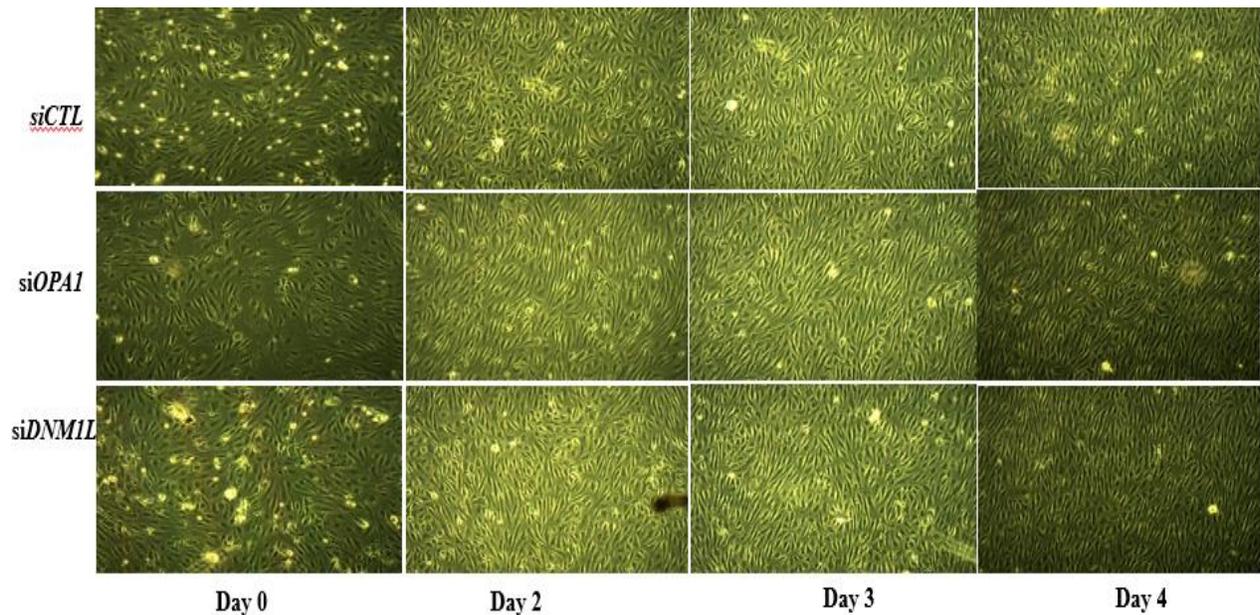


Figure 49 Inverted microscopic images for the alignment of the transfected HUVECs with siCTL as a control, siOPA1 to silence OPA1 and siDNM1L to silence DRP1, after 0, 2, 3, and 4 days of flow.

Unfortunately, at day 4, none of the three types of cells have shown any significant difference in the alignment of endothelial cells with flow direction (Figure 50). But these results were only for three different flow experiments which were not enough to reveal a good result.

Alignment after 4 days of flow exposure

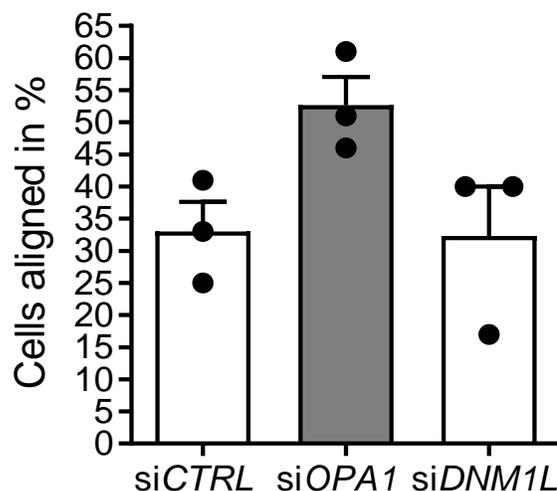


Figure 50 Analysis of the microscopic images captured after 4 days of the flow. Using ImageJ alignment tool, the percentage of cells aligned in each group was represented. n=3 independent experiments was analyzed. Ordinary one way anova was used as a statistical test.

6.2. Endothelium protein expressions in response to flow (to be continued)

HUVECs were transfected with siCTRL, siOPA1, and siDNM1L for 72 hours and then subjected to shear flow for 4 days. After 4 days, western blot was performed in order to test the effect of the mitochondrial dynamics proteins on the expression of different proteins in response to flow. After the validation of the efficient silencing of OPA1 and DRP1 (Figure 51A&B), we have tested for the the different protein expressions of eNOS, pAKT/AKT, ET-B and KLF-2 (Figure 51). However, these results are to be further validated with more experiments were we could also the expression of the vasodilation and inflammation receptor expressions. Thus, more flow experiments are being done at the moment to validate our hypothesis and also, some western blots will be performed after an exposure of HUVECs to flow for 1 day. These experiments will allow us to see the early changes in endothelial phenotypes in the absence or presence of regulated mitochondrial dynamics profile.

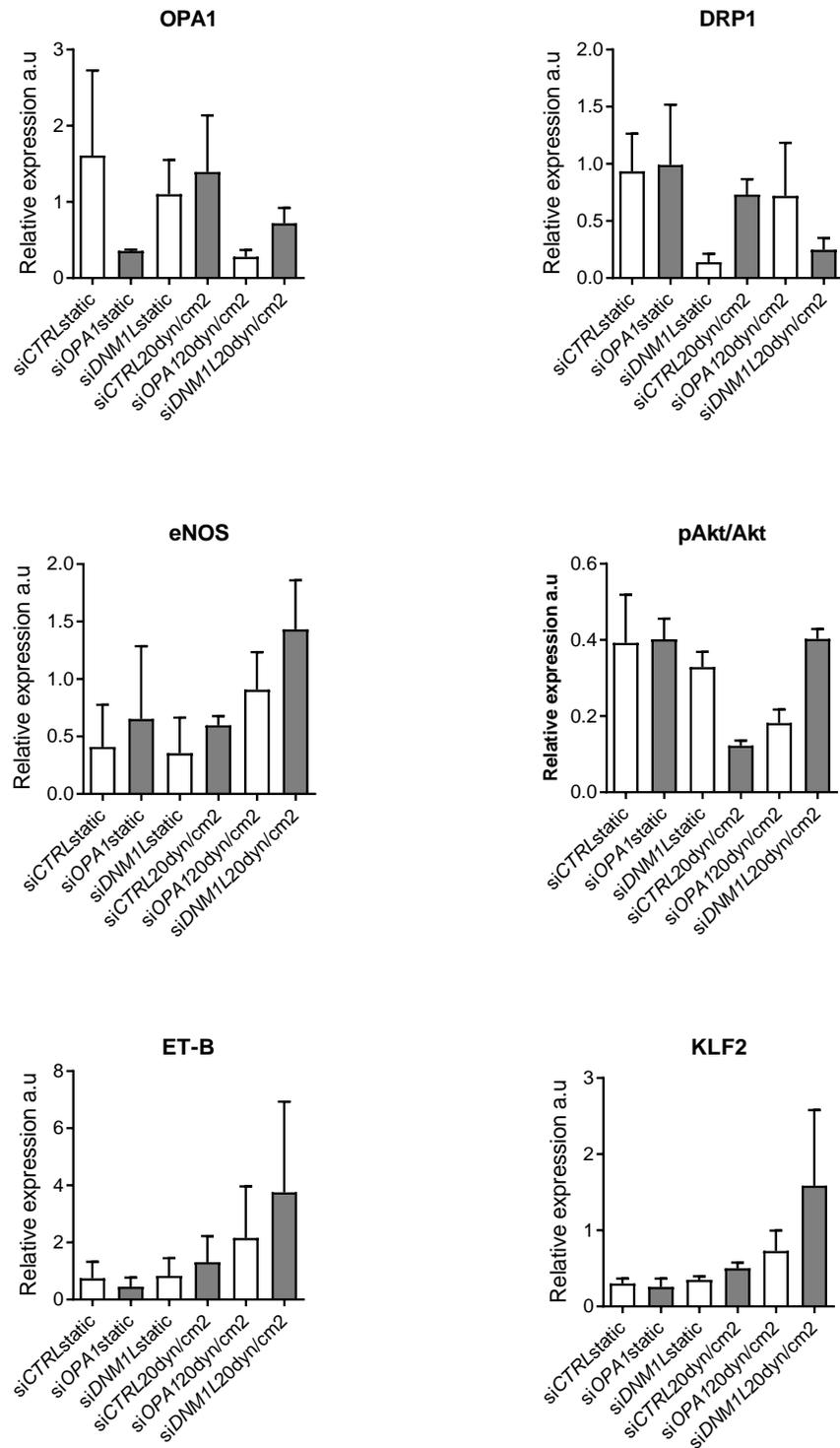


Figure 51 The different proteins relative expression after 4 days of flow exposure in the three groups of cells used.

7. Effect of flow on gene expressions of MS1 cells

In order to almost reach the same conditions that we have tested in vivo and the aorta is subjected to laminar and disturbed flow, we have tested for the effect of disturbed flow on endothelial cells. For that, MS1 cells were used to tune these experiments where these are secondary cell line isolated originally from mice pancreas and then immortalized using SV40 large T-antigen transduction to be secondary cell lines. MS1 cells were seeded in a 6-well plates and then allowed to shake on an orbital shaker of 210 (as a protective laminar flow condition) and 260 (as a disturbed flow condition) revolution per minute rpm for 24 hours.

In order to check for the effect of the two types of flow on endothelial MS1 cells. These cells were subjected to flow for 24 hours and then mRNA expression levels were analyzed after performing qRtPCR. Different genes, responsible for the expression of protective and aggressive proteins in response to flow, were studied. Our results revealed that MS1 cells under a flow of 210 rpm were able to significantly overexpress Cyp1b1 gene responsible for cytochrome P450 expression, and repress the expression of ET-1 by reducing the mRNA levels of Edn1 compared to static control (Figure 52). Experiment was done by Mrs. Jade Aurriere during her master 2 training period.

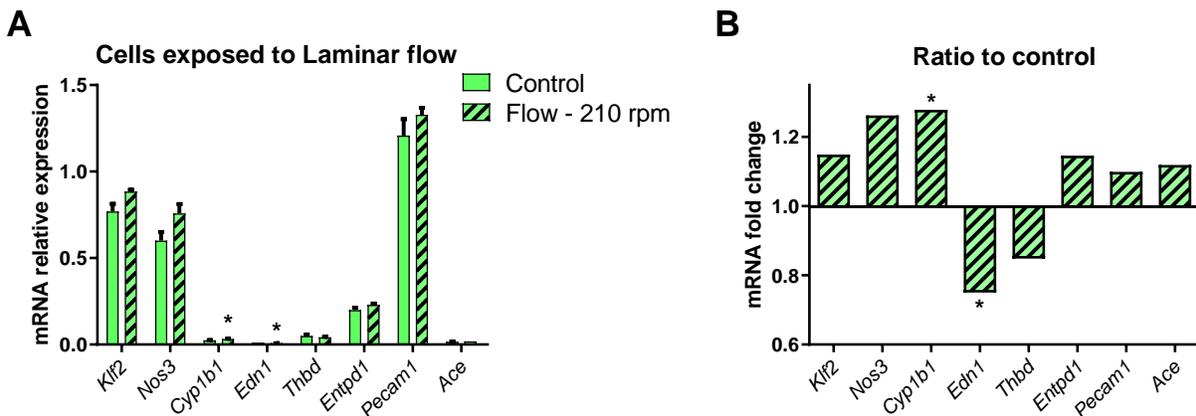


Figure 52 Expression of different genes in response to laminar flow of 210 rpm in MS1 cells. (A) mRNA expression levels of different protective and aggressive genes in the presence or absence of flow. (B) The ratio of the expression of these genes compared to the control static gene results. Statistical test used is Mann-Whitney test with $*P < 0.05$. $n=6$ from each group.

In contrast, MS1 cells exposed to disturbed flow of 260 rpm by the orbital shaker have shown different expression levels of these genes, where they have shown a reduction in the levels of protective genes and enhancement in the expression of aggressive genes. The mRNA relative expression levels have shown a

significant decrease in the levels of *Nos3* mRNA and an enhancement in the levels of *Edn1* mRNA levels in sheared cells compared to control static (Figure 53).

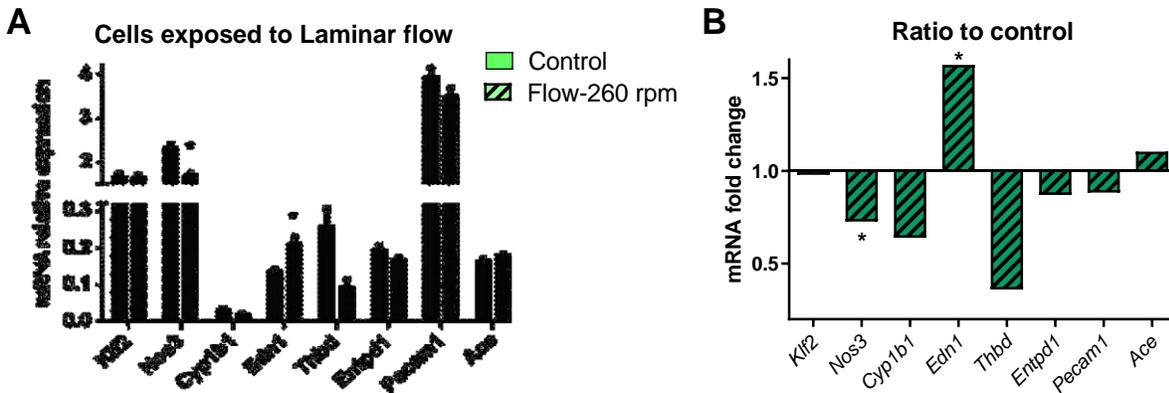


Figure 53 Expression of different genes in response to laminar flow of 260 rpm in MS1 cells. (A) mRNA expression levels of different protective and aggressive genes in the presence or absence of flow. (B) The ratio of the expression of these genes under 260 rpm of flow compared to the control static gene results. Statistical test used is Mann-Whitney test with $*P < 0.05$. $n = 6$ from each group.

8. Effect of disturbed flow on mitochondrial dynamics of MS1 cells

Then we have assessed the levels of mRNA relative expression of OPA1 and DRP1 proteins in both control (at static condition) and flow of 210 and 260 rpm. Our results revealed that laminar flow have no effect on the mRNA relative expression of mitochondrial dynamic proteins OPA1 and DRP1 at a flow speed of 210 rpm. However, MS1 cells subjected to 260 rpm have shown a disturbance in their mitochondrial dynamics balance where we have seen a significant decrease in the levels of *Opa1* mRNA relative expression levels of MS1 cells subjected to 260 rpm of flow compared to static control condition. This effect was reverted in the revelation of *Dnm1l* mRNA relative expression in MS1 cells subjected to 260 rpm flow compared to control static cells. Thus, we have obtained a significant enhancement in the levels of *Dnm1l* subjected to 260 rpm flow compared to control cells. Therefore, laminar flow hasn't affected mitochondrial dynamics as we have already shown in vivo mice models, but disturbed flow can dysregulate the balance in mitochondrial dynamics by shifting it more toward mitochondrial fission and reducing the levels of mitochondrial fusion levels. This was done by Jade Aurierre during her Master 2 training period.

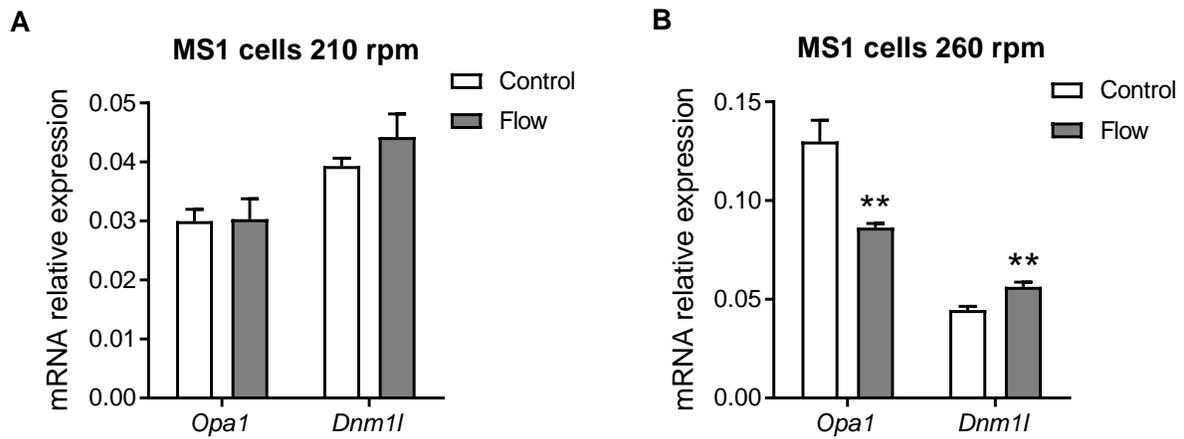


Figure 54 Effect of laminar shear stress and disturbed flow on mitochondrial dynamics. (A) The relative mRNA expression levels of mitochondrial dynamics proteins in response to a flow of 210 rpm (Laminar Flow) applied on MS1 cells by an orbital shaker. (B) The effect of disturbed flow of 260 rpm on the mRNA relative expression levels of mitochondrial dynamics proteins. Mann-Whitney test was used as a statistical test where ** $P < 0.01$.

Discussion

1. Introduction

The mitochondrion is known as the powerhouse of the cell; however, its role in endothelial cells extends far beyond generating ATP as they are more glycolytic in their energy sources. Thus, the mitochondrion in endothelial cells functions more as signaling organelles for their biochemical responses against several factors. An important character of mitochondria allowing it to powerfully perform its roles, is that they are highly dynamic organelles undergoing fission and fusion continuously. This character allows mitochondria to alter their shape interchangeably between elongated interconnected morphology and a fragmented disconnected morphology. Mitochondrial dynamics is regulated by different types of proteins including OPA1, MFN1, and MFN2 for mitochondrial fusion, and DRP1, Fis1, MFF, MiD49, and MiD51 for mitochondrial fission. In our study we focused on the two proteins OPA1 mitochondrial fusion and DRP1 mitochondrial fission protein.

2. Role of OPA1 in CVDs

The role of OPA1 in a number of cardiovascular and metabolic diseases has been investigated in several studies. That's why mitochondrial fusion targeting through the OPA1 protein was set as a potential therapeutic target in the treatment of several cardiac and metabolic clinical treatments (Figure 55).

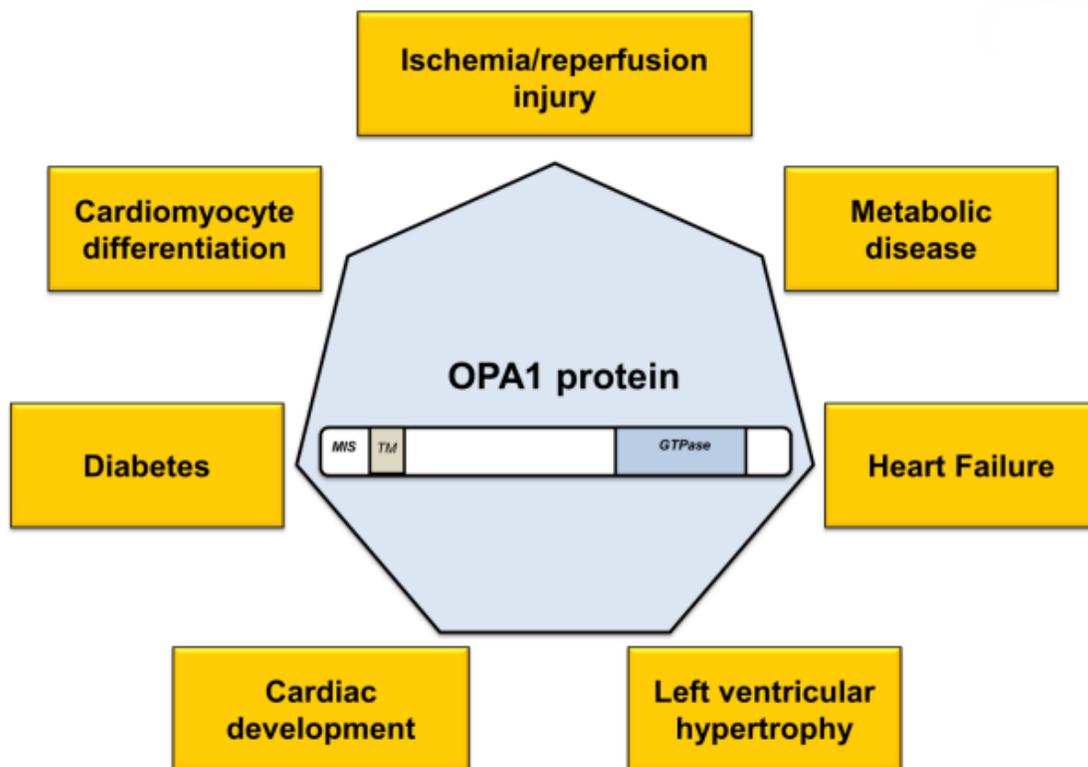


Figure 55 Roles of OPA1 in different cardiovascular and metabolic diseases (N. Burke and Hausenloy 2015, 1)

In patients with ischemic heart failure, the levels of OPA1 have been shown to be reduced with a reduction in mitochondrial fusion. This reduction was also confirmed in rat models without any change in the levels of Mfn1, Mfn2, DRP1 and Fis1, in induced ischemic heart failures. In addition, at the cellular level, H9C2 cells simulating ischemia have shown a reduction in the levels of mitochondrial fusion resulted in an increase in mitochondrial fission associated with an enhancement in cell apoptosis (L. Chen et al. 2009, 1). Besides, a recent study in 2016 have shown that the deficiency in OPA1 increases the sensitivity to cardiac ischemia reperfusion (Le Page et al. 2016a). Furthermore, in patients showing diabetes mellitus, an impaired mitochondrial fusion with low levels of OPA1 expression was investigated (Zorzano, Liesa, and Palacín 2009). All of these evidences show that mitochondrial dynamics plays a major role in most of the cardiovascular diseases, where a big part of these diseases result in an imbalance in mitochondrial dynamics or even some of these diseases are caused by the dysregulation of mitochondrial dynamics state. In order to accomplish this goal, we used first a mouse model of *Opal* haploinsufficiency as it reproduces well the human disease of optical atrophy that leads to the loss of vision (Sarzi et al. 2018).

3. Role of OPA1 in vascular reactivity

In the first part of our work we studied the influence of *Opal* haploinsufficiency on vascular functioning. As previous works have shown that the mitochondrial dynamic is regulated by flow and that shear stress mechanotransduction is mediated through the release of Ca^{2+} from the ER and the entry of extracellular Ca^{2+} , we studied flow-mediated dilation (FMD) in mesenteric arteries isolated from *Opal*^{+/+} and *Opal*^{+/-} mice. In *Opal*^{+/-} mice, we observed about 30% decrease in FMD compared to littermate OPA1^{+/+} mice. This change in FMD was most probably not due to a structural difference in the arterial wall as no significant difference in arterial diameter in response to pressure (pressure/diameter relationship) was recorded. In agreement, KCl and Phe-mediated contraction were not significantly difference between arteries from OPA1^{+/+} and OPA1^{+/-} mice. Finally, we also found no significant difference in Ach and SNP-mediated dilation *Opal*^{+/+} and *Opal*^{+/-} mice, suggesting that the reduction in FMD is not due a global loss of dilatory capacity of the endothelium or of the SMCs but a specific alteration in flow (shear stress)-mediated mechanotransduction.

Pre-menopausal women are better protected from cardiovascular diseases than males (Hayward, Kelly, and Collins 2000). Moreover, estrogen has been shown to play a role in the signaling pathways enhancing vasodilation, angiogenesis, and cell survival, but also reducing ROS production (Klinge 2008; 2017).

Although estrogens were shown to play a protective role against cardiovascular diseases (Iorga et al. 2017), its protective role in situation of altered mitochondrial dynamics was not yet investigated. The results obtained in female heterozygote *Opal*^{+/-} mouse were similar to those obtained in male *Opal*^{+/-} mice with

approximately the same percentage of decrease in FMD. Likewise, there was no difference in the response of mesenteric arteries to the vasoconstrictor agents KCl and Phe and to the vasodilator Ach. These results suggest that a decrease in mitochondrial fusion can lead to a selective change in the response of arteries to flow due to a change in an endothelial signaling pathway.

Different studies have shown a relation between mitochondrial dynamics and shear stress in cultured cells (Bretón-Romero et al. 2014; L.-H. Wu et al. 2018) but we were the first to study the effect of OPA1 signaling in the arterial response to changes in flow. Accordingly, our results of female mice revealed that OPA1 plays a role either upstream the protective signaling genomic and non-genomic pathways of estrogen in CVDs, or through an independent pathway from estrogen signaling in females (Deschamps, Murphy, and Sun 2010).

Nevertheless, to better conclude for the role of estrogen in the absence of mitochondrial fusion, we have generated an ovariectomized mouse model where the two ovaries were ligated for 10-14 days (Souza et al. 2019). After that, we have assured for the ovariectomization of these mice by taking the weight of the uterus that decreases more than 50% in OVX mice compared to WT control (Lemini et al. 2015). So, we analyzed the effect of mitochondrial fusion on vascular reactivity in female ovariectomized mice lacking OPA1 protein. Similarly, we tested for FMD, arterial diameter in response to pressure, vasodilation and vasoconstriction percentage, in OVX *Opal*^{+/-} mice and OVX *Opal*^{+/+} (used as control). Yet, our data obtained for analysis were not enough to have a clear conclusion regarding the differences between the two ovariectomized strains. Therefore, more experiments will be performed in addition to some other experiments to determine the right position for *Opal* in the signaling of estrogen in the protection of endothelial cells physiological behavior in response to flow (Results were not shown in the Results part due to the lack of revealed data).

4. Mitochondrial Dynamics and Endothelial Functioning

As endothelial cells are the first cells exposed and in contact to flow, they respond to shear factors differently depending on the type of flow. But, the type of flow depends on the arterial zone and curvature morphology. Under physiological conditions, the endothelial cells possess several characteristics that determine their normal functioning. In the vascular tree the main function of endothelial cells is flow mechano-transduction where they are the sites of conversion of the mechanical stress into intracellular biochemical signals. Then, vascular SMCs are able to induce a change in the vascular structure to maintain vascular homeostasis. Recently, it has been shown that a perturbation in mitochondrial dynamics through silica nanoparticles stimulation of HUVECs, leads to a reduction in the mitochondrial DNA copy number and in mitochondrial biogenesis. In this study they have shown that under stress conditions, HUVECs

showed a reduction in mitochondrial ROS, a decrease in mitochondrial membrane potential Ψ_m , impairments in the synthesis of ATP, and an elevated levels of intracellular calcium (Caixia Guo et al. 2018). Besides, an endothelial dysfunction have been shown recently to be a consequence of alterations in mitochondrial dynamics and mitochondrial ROS production (Lahera et al. 2017). Given this context, targeting the mitochondrial dynamics of endothelial cells appears to be an attractive study that allows us to check for the endothelial functioning and have a clear idea for the reason of obtaining a decreased FMD. Herein, we have silenced the two key proteins of mitochondrial fission and fusion processes in HUVECs.

5. Mitochondrial dynamics and calcium signaling

A common signaling second messenger in endothelial cells is calcium. Calcium signaling is a key player in the release of different factors that respond to flow and other indirect dilators in ECs. These act indirectly on ECs by inducing a huge efflux of intracellular calcium from the ER to the cytosol, which in turn activate different signaling pathways that allows the release of NO, EDHF, and PGI₂ (Figure 56).

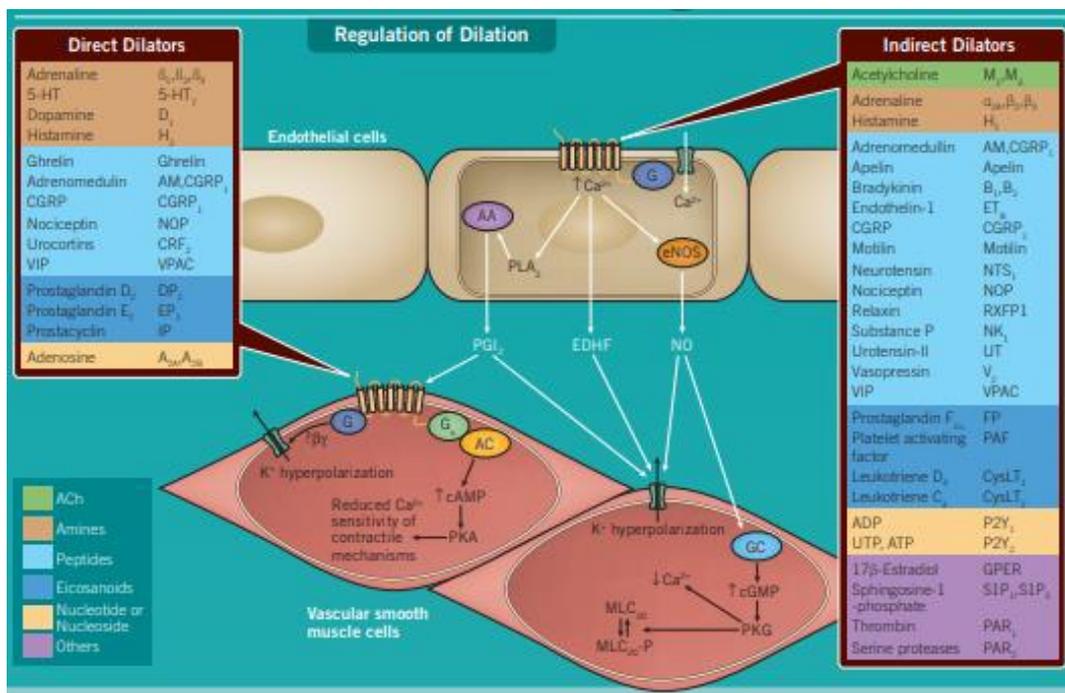


Figure 56 Direct and indirect dilators calcium signaling in endothelial cells for the regulation of dilation process in arteries (Maguire and Davenport 2017)

Our data on FMD suggest an endothelial dysfunction on the pathway activated by flow (shear stress). To better understand the endothelial changes in OPA1 deficient arteries, we performed calcium measurements on cells lacking OPA1 or DRP1 proteins.

When an agonist stimulates a calcium signaling cascade, it first targets the ER that is the major Ca^{2+} store in the cytoplasm. However, the mitochondria play an important role in calcium signaling mainly because of its positioning near the ER. This leads to an interplay in calcium signaling between ER and mitochondria. When a ligand binds to a cell surface receptor (GPCR for example), it activates the IP3 pathway which in turn activates IP3R and stimulates the release of calcium from the ER. The calcium is released to the cytosol through IP3R channels and ryanodine receptors (RyR), which are mainly localized to the points of interaction between ER and mitochondria, sites called MAMs (mitochondrial associated membranes). Intracellular calcium waves from the cytosol enter to the mitochondria through voltage dependent anion channels VDAC or calcium uniporters CU to enhance the activity of ETC that leads to the increase in ROS production. Mitochondrial ROS liberated to the cytosol stimulates again the ER stores to release more Ca^{2+} that acts as a second messenger in the cell to accomplish several functions (Görlach et al. 2015). However, under certain conditions, the high release of ROS and calcium load in the mitochondria due to a mitochondrial dysfunction, leads to the opening of the mPTP but also to the rupture of the mitochondrial membranes that shifts the cells more toward apoptosis signaling pathways (Rizzuto et al. 2012). In this respect, the absence of mitochondrial fusion in HUVECs leads to a reduction in the levels of intracellular Ca^{2+} concentrations in response to histamine stimulation, where during aging, the deficiency in *OPA1* was shown to increase the levels of mtROS production with a decrease in the levels of mitochondrial anti-oxidant enzymes (Tang et al. 2009).

On the contrary, we observed a reduction in the levels of intracellular calcium in response to histamine as mitochondrial dynamics proteins were reduced. These results are not compatible with the results obtained in vivo where we have no significant difference between mesenteric arteries from the two strains after stimulation with Ach. But, as we have revealed lower calcium levels in cells lacking OPA1 and DRP1, this will allow us to postulate a hypothesis that these lower levels will reduce the activity of eNOS and liberate lower levels of NO that will decrease the dilation regulatory process in *Opal* deficient mice. However, HUVECs stimulated with ATP haven't shown any significant difference to HUVECs lacking mitochondrial dynamics proteins. This could be referred to the fact that ATP is stimulating P2Y receptors whereas Histamine stimulates H1 receptors on endothelial cells. Although both types of receptors belong to GPCR family, the dispersion and quantity of both receptors are different (Kaur et al. 2017). But, Hatoum et al. have shown in 2005 that in microvessels, H_2O_2 is produced in high levels in response to Ach but this is not an EDHF (Hatoum et al. 2005).

6. Role of mitochondrial dynamics in endothelial functioning

An important character of endothelial cells is their ability to migrate in response to a variety of pathological and pathophysiological processes. These processes include wound healing, angiogenesis, vascular repair,

and atherosclerosis. Thus, an important point in our project is to study the effect of mitochondrial dynamics in one of the most important functions of endothelial cells. Although the mitochondria have been implicated in different endothelial functioning, oxidants are categorized as important regulators of endothelial cell migration and adhesion (Förstermann 2008). The mtROS which is generated from several signaling pathways and known as cellular respiration byproduct, plays an important role as an intracellular signaling mediator in response to different stimuli (D. X. Zhang and Gutterman 2007). Despite this, the understanding to the precise effect of mitochondrial dynamics regulation on endothelial cell migration is scant. Our study here reports the effect of the dysregulation of mitochondrial dynamic proteins on the endothelial cells migration function.

It has been shown that an increase in the intracellular calcium levels can stimulate ECs migration (Moccia, Tanzi, and Munaron 2014). Our experiments have revealed a decrease in intracellular Ca^{2+} levels in ECs lacking mitochondrial dynamic proteins of OPA1 and DRP1. This decrease in the intracellular calcium levels was associated with a decrease in the migration capability of these cells, which provides a good evidence that an alteration in the balance of mitochondrial dynamics leads to endothelial cell dysfunction through a signaling pathway mediated by intracellular Ca^{2+} levels.

7. Effect of shear stress on mitochondrial dynamics

Large arteries are exposed to different flow patterns as they are highly variable in structure and morphology. Thus, the endothelium of these arteries responds differently in the different zones of the aorta. In the regions protected from atherosclerosis, these ECs are exposed to pulsatile unidirectional shear stress that has generally a flow of 15-70 dynes/cm² under physiological conditions. However, the flow is oscillatory varying close to zero and less than 10-12 dynes/cm² in the regions with curvatures, bifurcations and branching points of large arteries, where these zones are considered as atheroprogenic (Chatzizisis et al. 2007b). These different patterns can differentially modulate the gene expression of the endothelial cells from the different aortic zones. LSS increases NO production by activating eNOS (Kuchan and Frangos 1994), and increase MnSOD level (Ai et al. 2008), in order to protect from atherosclerotic lipid deposit. However, an increase in the ROS production was observed in ECs subjected to oscillatory flow (Hsieh et al. 2014). Thus, the perturbation in flow leads to an increase in oxidative stress levels and ROS production in the endothelial cells.

Although oxidative stress is known to activate fission and impair mitochondrial fusion in endothelial cells, the effect of flow on the mitochondrial dynamics of endothelial cells in the different aortic zones is not yet well identified. In our study we found that in the zones subjected to oscillatory low flow levels, mitochondrial fission is favored over mitochondrial fusion compared to the other aortic zones. The

difference in the mitochondrial dynamic mRNA expression occurred mainly in the aortic arch AA which is subjected to the lowest and the most oscillatory flow pattern. In this zone, we have obtained a significant decrease in *OPA1* mRNA expression levels compared to abdominal and thoracic aorta where laminar shear stress is the LSS predominates. However, *Mfn2* levels were increased in the AA compared to AAo, with a decrease in its level in AAo compared to AbAo. This doesn't change our conclusion regarding the mitochondrial fusion reduction in the zones of low OSS because it has been shown that OPA1 and *Mfn1* are the proteins essential for mitochondrial fusion process regardless to the presence or expression of *Mfn2* (Cipolat et al. 2004).

On the other hand, mitochondrial fusion seems to be more complicated than mitochondrial fission, where in mitochondrial fission only *Dnm1* and *Fis1* are required. In HUVECs, a decrease in the expression of *DRP1* and *Fis1* was associated with an increase in the resistance to oxidative stress in senescent cells. Besides, this year (may 2020), Kim and Roy have shown that *DRP1* down regulation is more effective in reducing mitochondrial fragmentation than *Fis1* downregulation (D. Kim, Sankaramoorthy, and Roy 2020). This importance of *DRP1* over the importance of *Fis1* allows us to suggest that mitochondrial fission is higher in AA even if there was no significant change in the levels of *Fis1*.

Interestingly, in the mesenteric arteries which are small arteries that do not show different morphological curvatures in structure, no change in mitochondrial dynamics mRNA expression was observed. These results are in agreement with our results obtained in AbAo and ThAo where LSS predominates. Besides, mesenteric arteries from mice, can't develop atherosclerotic plaque due to their small size where macrophages cannot enter these arteries. However, MA with LF have shown a reduction in the levels of eNOS expression compared to HF and NF arteries, this clearly explains the role of NO in the vasodilation of these arteries where eNOS activity is less stimulated in LF arteries. In contrast, these arteries express more ET-1 which is needed for their contraction mechanism. These results are in agreement with previous works by our laboratory, where we have shown that an increase in the flow in mesenteric arteries from rats was associated with an increase in the levels of eNOS expression and endothelial functioning (Tarhouni et al. 2014).

Indeed, different transgenic and knock out mouse models were developed in order to study cardiovascular diseases. Among these are the two mice models of Apolipoprotein E ApoE and low density lipoprotein receptor *Ldlr* KO mice, where these two models are used for their ability to develop atherosclerotic plaque and hyperlipidemia (Laakkonen and Ylä-Herttuala 2016). For that in our study we have chosen to work with *Ldlr*^{-/-} mice and feed them with HFD to allow them to develop atherosclerosis. Mice fed with HFD have shown the same result where mitochondrial fusion mRNA expression levels were all decreased due to the increase in the variability of flow pattern in these mice. In contrast, these mice have shown the opposite

result for the expression of Drp1 and Fis1 were they have significantly decreased their expression levels of Fis1 and not Dnm11. The decrease in mitochondrial fission process could be referred to the pathological situation that these mice present. So that, after 4 months of HFD, these mice developed lipid deposits in the wall of the arteries with an early phase of endothelial dysfunction. Thus, under pathological conditions, it is not only the structural pattern of the aorta which is altering the flow pattern but also the deposited plaque that increases the variability of the flow and thus alters the gene expression in endothelial cells.

8. Mitochondrial Dynamics and Atherosclerosis

Atherosclerosis is the deposition of lipid fatty material in the walls of medium and large arteries leading to a partial or complete blockade of blood flow. It is a main cause of CVDs that occurs in different stages starting from the injuries caused by the blood flow.

Nonetheless, the role of mitochondrial fission or fusion protein as a therapeutic target for atherosclerosis, is still not quite clear. A recent study, in July 2020, have demonstrated the role of CoQ10, a component of the mitochondrial respiratory chain complex. This was the only study implicating a role of mitochondrial OPA1 protein in the development of atherosclerosis, where CoQ10 exerts its effect in the protection from atherosclerosis through the AMPK-YAP-OPA1 pathway. The therapeutic effect of CoQ10 was reduced after the reduction in the expression of OPA1 through the silencing of YAP and AMPK. After the silencing of AMPK, an energy sensor and inflammatory signaling marker, they revealed an inhibition of the phosphorylated levels of YAP, an activator of the STAT3 and vascular remodeling. Definitely, the inhibition of YAP activity leads to a reduction in OPA1 levels that consequently decreases ATP production, increases inflammatory responses and elevates ROS production. As they have showed in the same study that a reduction on the activity of CoQ10 increased the levels of atherosclerotic plaque, we can link OPA1 expression with the protection from the development of atherosclerotic plaque (Xie et al. 2020).

But, complete OPA1 KO was shown to be lethal in the early stage of development where *Opa1*^{-/-} embryos die by 13.5 days post coitun (Davies et al. 2007). This reveals the importance of OPA1 during development and that mitochondrial fusion plays an important role during embryogenesis (Davies et al. 2007). For that, we investigated a heterozygous deficient *Opa1* model (*Opa1*^{+/-} model) (Le Page et al. 2016b). Mice lacking OPA1 (*Opa1*^{+/-}) had a higher level of lipid deposit (or fatty streak deposit) which is a most probably the consequence of the endothelial dysfunction that we found in these mice. Although this effect was not observed in females OVX female *Opa*^{+/-} mice had also a larger level of fatty streak deposit suggesting that in the absence of estrogen protection both male and female mice are more prone to atherosclerosis with a reduced level of OPA1. In male and female WT and *Opa1*^{+/-} mice, we did not detect any significant difference in other main parameters that are affected by HFD. Estrogen have been shown to protect arteries

from atherosclerosis through activation of the estrogen receptor alpha (ER α)-dependent activating function 2 (Billon-Galés et al. 2011).

A complete KO of Drp1 was shown to embryonically die after day 11.5 where Drp1-null embryonic mice fail to develop the normal apoptotic signals during neural tube formation. These mice showed normal intracellular ATP levels and their death wasn't referred to an energy deprivation (Wakabayashi et al. 2009). For that, we investigated a heterozygous deficient Drp1 model (*Drp1*^{+/-} model) (Wakabayashi et al. 2009).

By contrast with *Opal*^{+/-} mice, fatty streak deposits were reduced in male DRP1^{+/-} mice compared to WT mice. *drp1*^{+/-} mice also showed a reduction in glycemia level without change in any other HFD-affected parameters. Mitochondrial fission protein was shown to be important in high-glucose induced apoptosis (Bhatt et al. 2013), and mitochondrial fission contributes to diabetes-induced endothelial dysfunction (Shenouda et al. 2011). Besides, Wang et al have shown that an inhibition of Drp1 is protective for endothelial cells against atherosclerosis. In this study they have shown that metformin, an antidiabetic drug, protects from atherosclerosis in vivo via AMPK-mediated blockade of mitochondrial fission driven by the phosphorylation of DRP1 at 616 in diabetic mice (Q. Wang et al. 2017). These findings, in agreement with our results, strongly suggest that the inhibition of DRP1 activity could prevent atherosclerotic plaque formation. More research is necessary to further understand the signaling pathway by which DRP1 in the protection from atherosclerosis.

Due to the known protective role of estrogens in CVDs, we have also studied the effect of DRP1 reduction in the presence of the female hormones. To date, not study is known regarding the effect of Drp1 in the presence of female hormones or estrogen. Herein, we have highlighted an important effect of estrogen on the activity of DRP1. While we have recorded an inflammatory role of DRP1 in male mice increasing endothelial dysfunction and atherosclerosis, the presence of estrogen reduced the atherosclerotic plaque deposit in WT females but not in *Drp1*^{+/-} females. Hence, this observation could suggest that the protective effect of estrogen is partly associated with the presence of Drp1 or with the high mitochondrial quality control processes. It has been shown that there is a direct interaction of ER β with AKAP1, a mitochondrial resident protein, which leads to the inhibition of mitochondrial fission and increase in OXPHOS. Thus, we could hypothesize that the protective role of estrogen is mediated through its interaction with ERs and reduction in DRP1 activity (Sarkar, Jun, and Simpkins 2015). However, another hypothesis could be postulated to the threshold of reduction in the activity of DRP1, as in *Drp1*^{+/-} we haven't seen a protective activity. Therefore, further studies must be done in order to find the pathway by which Drp1 is affecting estrogen protection. Moreover, it is important to increase the number of mice used in the study of atherosclerosis in *Drp1* females, but also to ovariectomize DRP1 mice is a better way to explore the signaling pathway by which estrogens are acting through DRP1.

9. Role of Mitochondrial Dynamics in the response of ECs to flow

Cells respond to flow by a change in their structural and transcriptional behavior to accomplish their mechano-transduction functioning. Studying the endothelial cell alignment is a good way to check the phenotypic properties of these cells. For ECs alignment, the mechanical signals are used to stimulate cytoskeletal and transcriptional responses to reform the artery size and structure. Previous studies have shown the effect of shear stress on the alignment of endothelial cells but as the mitochondria are anchored to the cytoskeleton, we hypothesized that the change in mitochondrial number and size by fission/fusion events can alter the alignment function of ECs. Besides, as we have obtained a difference in the migration capability of migration in cells with a defect in their metabolic dynamics balance, we hypothesized that a change in mitochondrial dynamics alters the alignment of endothelial cells in response to flow.

A good marker of the early phase of atherosclerosis is the mal-alignment of ECs with the direction of flow in the zones of perturbed flow. In contrast, in the zones subjected to LSS, ECs shows a good alignment with the flow pattern that makes a resistance to atherosclerotic plaque formation (Baeyens et al. 2014). Our experiments did not show any significant difference in the directionality of the ECs between control and siRNA treated cells, after laminar shear exposure for 4 days. This observation could suggest that mitochondrial dynamics proteins are not involved in the alignment signaling pathway induced by LSS. Although determining the levels of expression of proteins involved in vascular responses to flow is an important level in cell alignment experiments, the number of laminar flow experiments was not enough to obtain a clear significant conclusion.

On the other hand, shear stress induced on ECs by the action of orbital shaker were well characterized, whereby cells seeded on a well with 210 rpm were subjected to a shear of 11.1 dyne/cm^2 in the periphery of the well and to 4.8 dyne/cm^2 in the center of the well (Dardik et al. 2005). However, in order to activate also the endothelial cells at the center with a harsh stressful velocity, it was necessary to use a velocity higher than 240 rpm. Hence, in our project we used two distinct shaking velocities of 210 rpm and 260 rpm and we studied the effect of these two shear levels on ECs mitochondrial dynamics and vascular reactivity genes. In accord with previous studies, these changes in ECs responses and the fact that 260 rpm repressed the eNOS genes and expressed the ET-1 genes, we demonstrated that 210 rpm was kind of athero-protective flow and 260 was considered as an oscillatory disturbed flow (Resnick et al. 2003). At a shear level induced by 210 rpm, the mitochondrial dynamics were not changed compared to the control which is equivalent to the cells at static conditions. In contrast, with a higher stress induced by 260 rpm, the response of the cells is altered, and it can be assimilated to a disturbed flow. With this shear stress, the balance of mitochondrial dynamics shifts more towards mitochondrial fission. This is in agreement with the literature where they detected an increase in fission observed in response to severe shear stress (Giedt et al. 2012). Therefore, in

this cellular model the oscillatory disturbed flow shifted mitochondrial dynamics balance more toward fission. However, it remains important to validate these results on HUVECs and apply disturbed flow using another invitro precise model. Also, the cells during these experiments were seeded in all the wells surface, which clearly indicates a high variability in the shear applied to the cells of the same well (Dardik et al. 2005).

Conclusion & Perspectives

This work was conducted to:

1. Understand the role of mitochondrial dynamics proteins in the endothelial cells layer of the arteries.
2. Study the effect of different flow patterns on mitochondrial dynamics of large arteries.
3. Investigate the effect of mitochondrial dynamics proteins in the response of endothelial cells to flow.
4. Demonstrate the role of mitochondrial dynamics proteins in atherosclerotic plaque formation.

Firstly, our results highlighted the importance of mitochondrial fusion in the response of arteries to flow regardless to the protective effect of female hormones. We showed that a partial fusion inhibition significantly reduced the response of mesenteric arteries to flow while the integrity of these arteries is not affected by mitochondrial fusion reduction. We were able in this study to show that the difference in the vascular homeostasis phenomena induced by alteration in mitochondrial dynamics is referred to as a signaling pathway responding only to the flow in our ex-vivo experiments. However, our in vitro calcium and cell migration assays clearly showed that mitochondrial dynamic proteins act upstream the intracellular calcium efflux signaling regulatory pathway. Subsequently, the reduction in mitochondrial dynamics proteins was associated with endothelial dysfunction. These data mark, on one hand, the importance of mitochondrial fusion in the response of the arteries to flow and on other hand, a potential implication of mitochondrial dynamics proteins in the early phase of atherosclerosis characterized by an early phase of endothelial dysfunction. Nonetheless, it could be interesting to investigate the role of mitochondrial fission in the response of mesenteric arteries to flow and other agonists, also, as mentioned beforehand, it would be interesting to ovariectomize some mice to better understand the role of estrogens if they have a compensatory role of mitochondrial dynamics loss in balance.

Secondly, in this study we were the first that to demonstrate the levels of expression of mitochondrial dynamic proteins in different aortic zones, where we showed a reduction in the levels of mitochondrial fusion and an increase in mitochondrial fission in the zones subjected to disturbed shear stress. We also confirmed that the change in flow intensity, only, cannot alter mitochondrial dynamics proteins expression but also a difference in shear stress due to morphological geometry is essential. Besides, diameter changes in small arteries, due to modification in the speed of flow, are maintained by the changes in expression levels of ET-1 and eNOS induced by flow.

Thirdly, we confirmed that endothelial cells subjected to laminar flow do not show any change in the alignment regardless to the balance mitochondrial dynamics proteins. These results confirm the results of FMR where mitochondrial dynamics proteins haven't shown any implication in the arterial diameter changes. Using the Ibidi flow system, we are able to mimic the same flow patterns that exist in large arteries.

For that, our experiments were performed under laminar flow conditions using this system, but it remains important to perform the same experiments under disturbed flow conditions. This would be either confirming or contradicting the results obtained on MS1 cells that we used on the experiments of orbital shaker. However, the results obtained from the orbital shaker experiments clearly indicated that under disturbed flow condition the ECs reduces its mitochondrial fusion events and increases mitochondrial fission events compared to cells at static conditions. These data mark the importance of mitochondrial dynamics in the response of endothelial cells to disturbed flow where the zones susceptible to atherosclerotic plaque exists.

Finally, as atherosclerosis is a disease associated with endothelial dysfunctions, we were able to show a protective role mitochondrial fusion OPA1 protein from the development of atherosclerotic plaque. This protective effect was conditioned by the absence of estrogens in female mice. In contrast, an atheroprogenic role was assigned to the presence of DRP1 in male mice associated with an increase in glycemic levels. These results were reversed in females where DRP1 showed a protective role from atherosclerotic plaque in the presence of estrogens. Thus, in female mice, mitochondrial dynamics proteins are important to maintain the protective effect of estrogens, whereas mitochondrial fusion is important for the protection from atherosclerosis in male mice. But, as mentioned before, it remains important to explore the signaling pathway by which mitochondrial dynamics balance is implicated in the protection from atherosclerosis.

To summarize, this project was important to conduct the effect of mitochondrial dynamics on the response of endothelial cells to shear stress induced by blood flow. Also, the alteration in the balance of mitochondrial dynamics proteins leads to endothelial dysfunction, an early marker of atherosclerosis. Indeed, our work proves furthermore that mitochondria and mitochondrial dynamics proteins are potential therapeutic targets in treating atherosclerosis.

Annexes

Article 1:

**Flow-mediated outward arterial remodeling
in aging**

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Article Type:	VSI: Growth and Remodeling
Keywords:	blood flow, Arterial outward remodeling, aging, estrogens, gender
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Abstract:	After intense vascular growth during embryogenesis and somatic growth, the vascular tree becomes relatively quiescent in adulthood. It nonetheless remains able to adapt to changing situations, such as exercise or pregnancy, which both require increased blood flow. In addition, in ischemic disorders, the ability of the vasculature to adapt or remodel determines the quality of the recovery. Healthy aging is characterized by a decrease in the capacity of the vasculature to adapt to changes in hemodynamic load. This generally does not have major adverse consequences. The capacity of arteries to remodel in response to flow becomes progressively less with age, and this reduction occurs later in females. Estrogen and its alpha receptor (ER α) play a role in this remodeling. This activity is associated with many protective effects of ER α against cardiovascular and metabolic disorders. With age, the dilatory pathways, mainly the NO pathway, become less active as a result of an increase in inflammation and oxidative stress. Importantly, mitochondria, which have a limited role in endothelial cells, may become more active with age, especially when associated with risk factors such as hypertension or diabetes, thereby contributing to the production of reactive oxygen species that reduce the bioavailability of NO
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Flow-mediated outward arterial remodeling in healthy and pathological aging

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Abstract

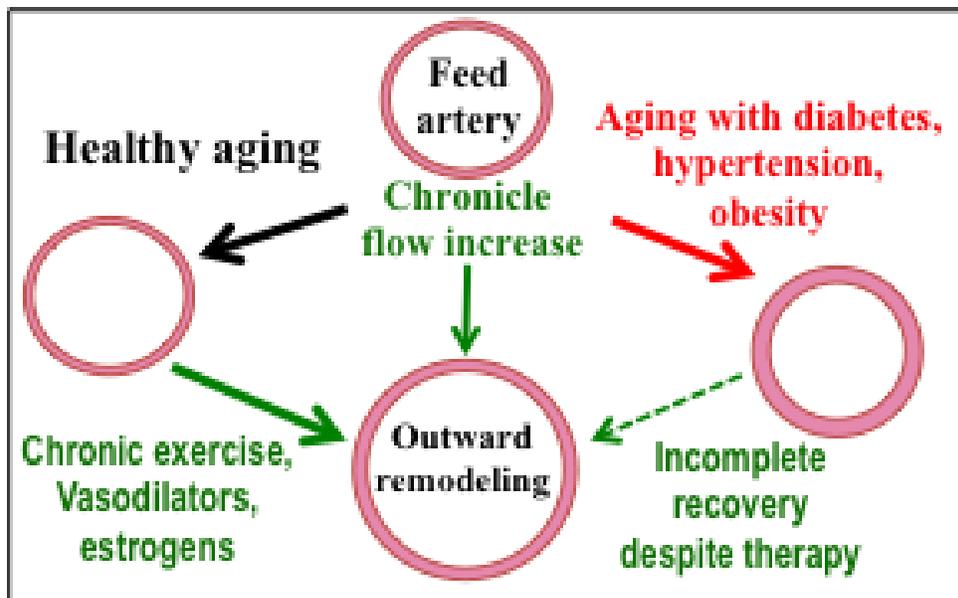
After intense vascular growth during embryogenesis and somatic growth, the vascular tree becomes relatively quiescent in adulthood. It nonetheless remains able to adapt to changing situations, such as exercise or pregnancy, which both require increased blood flow. In addition, in ischemic disorders, the ability of the vasculature to adapt or remodel determines the quality of the recovery. Healthy aging is characterized by a decrease in the capacity of the vasculature to adapt to changes in hemodynamic load. This generally does not have major adverse consequences. The capacity of arteries to remodel in response to flow becomes progressively less with age, and this reduction occurs later in females. Estrogen and its alpha receptor (ER α) play a role in this remodeling through its nuclear activating function AF2. This activity is associated with many protective effects of ER α against cardiovascular and metabolic disorders. With age, the dilatory pathways, mainly the NO pathway, become less active as a result of an increase in inflammation and oxidative stress. Importantly, mitochondria, which have a limited role in endothelial cells, may become more active with age, especially when associated with risk factors such as hypertension or diabetes, thereby contributing to the production of reactive oxygen species that reduce the bioavailability of NO.

Keywords: blood flow, endothelium, collateral growth, aging, estrogens, mitochondria

Highlights

Flow-mediated outward remodeling (FMR) is involved in post-ischemic revascularization
FMR is governed by shear stress and the immune system
Estrogens can activate the NO pathway involved in FMR
FMR is reduced in healthy aging, but it can be restored pharmacologically
Age and pathological risk factors abrogate FMR, often irreversibly

Graphical abstract



1. Introduction

Flow-mediated outward remodeling (FMR) promotes the growth of pre-existing arteries. It occurs in somatic growth, along with angiogenesis and arteriogenesis ¹. In adulthood, uterine arteries undergo regular outward remodeling during the menstrual cycle and a pronounced enlargement in diameter during pregnancy ². In pathological situations, such as diabetes ³⁻⁶ and hypertension ^{7, 8}, FMR and thus the growth of collateral arteries is altered. Recovery after an ischemic event is, therefore, more difficult when associated with these health risk factors. Furthermore, as has been shown in rats ⁹ and mice ¹⁰, aging itself is also associated with reduced FMR or collateral growth. Similarly, aging has been reported to be a critical issue in elderly individuals for the management of revascularization ¹¹. Nevertheless, as has been shown in rats, the pathway involved in remodeling remains efficient and can be rescued by the administration of a vasodilator drug ¹². This suggests that while the threshold for the response to flow is shifted, the pathway itself may not be affected ¹². Interestingly, the extent of the decrease in FMR in old female rats is much less compared to age-matched males ^{13, 14}. Indeed, it is well known and documented that pre-menopausal women have a lower incidence of cardiovascular diseases compared to men. This low incidence of cardiovascular diseases has been largely attributed to the protective effect of estrogens, which exert many beneficial effects in arteries, including arterial dilation, acceleration of healing after arterial injury, and atheroprotection ¹⁵. Thus, estrogens are thought to reduce the effects of vascular aging. Indeed, estrogens are also potent regulators of mitochondrial functions and they exert a protective effect on mitochondria ¹⁶. However, endothelial cells (ECs), which sense shear stress due to blood flow, contain a low number of mitochondria and they depend mainly on glycolysis for ATP production ¹⁷. This results in ECs having a low level of reactive oxygen species (ROS) as aerobic oxidation is limited in these cells. This is important as ROS play

a major role in endothelial dysfunction ¹⁸. In addition, ECs are not exposed to a hypoxic environment during angiogenesis under ischemic conditions, and glycolysis produces lactate, which is a strong activator of angiogenesis ¹⁹. Thus, a role for mitochondria in flow (shear stress) sensing, which is the trigger for FMR, is unlikely, although the key role of oxidative stress in FMR ²⁰ could nonetheless be associated with mitochondrial functioning. On the other hand, excessive oxidative stress can also involve mitochondrial ROS production, which could impair FMR in pathological situations, such as diabetes ³⁻⁶ or hypertension ^{7, 8}. Interestingly, mitochondria are thought to be the biological clock of cells. The American Heart Association has issued broad overview of the key role of mitochondria in cardiovascular physiology that provides insight into how mitochondrial defects contribute to cardiovascular diseases ²¹.

2. Revascularization: angiogenesis, arteriogenesis, collateral growth, and the role of flow-mediated outward remodeling

Post-ischemic revascularization is the result of several processes that occur simultaneously or sequentially ¹. First, angiogenesis is triggered by the ischemia and starts with the sprouting of preexisting capillaries. Arteriogenesis then allows capillaries to form arterioles after stabilization of the sprouts by smooth muscle cells and pericytes. Arteriogenesis hence increases the arteriolar density, which then results in more blood being available to feed the new capillaries. In parallel, collateral artery growth results from the outward remodeling of preexisting arteriolar anastomoses, which had little flow. Collateral growth increases the number of conducting arteries. Finally, upstream arteries also become subjected to increased blood flow and undergo outward remodeling,

leading to an increase in their diameter. Ultimately, blood flow is restored and the tissue recovers normal functioning (Figure 1).

An increase in blood flow is the main trigger for the outward remodeling that occurs in arteriogenesis, collateral growth, and for the increase in diameter of larger feeding arteries.

3 - Flow-mediated remodeling in somatic growth and other physiological processes

During somatic growth, the diameter of blood vessels increases along with vessel wall hypertrophy so that mechanical strength and vasomotor tone allow proper control of blood flow to tissues with growing needs ²². As in pregnancy, this remodeling has a number of features in common with the remodeling that occurs in post-ischemic revascularization. It is, however, mainly governed by growth factors and hormones at a level not observed later in life.

Remodeling of the uterine arteries allows for the pronounced increase in uterine blood flow that occurs during pregnancy (for a review, see Mandala & Osol ²). This is three-dimensional remodeling with enlargement in the diameter along with a substantial axial elongation and changes in vessel biomechanical properties, although wall thickening remains limited. Both local and systemic humoral systems regulate uterine artery remodeling, with a major role for shear stress and steroid-induced upregulation of endothelial NO production ²³. The placenta also activates this remodeling by producing VEGF or PlGF, with venoarterial communication as a plausible pathway for the placenta to control the outward remodeling of afferent arteries ². Although estrogens control outward remodeling in peripheral arteries ²⁴, their role in the remodeling of uterine arteries and veins remains uncertain.

Exercise induces a strong angiogenic stimulus in the active muscles where the capillaries are functionally recruited due to the increase in blood flow ²⁵. Indeed, exercise increases blood flow (shear stress), blood pressure, and wall stress, thereby leading to outward hypertrophic remodeling. Numerous studies have demonstrated that the direct hemodynamic impact of exercise training on the health of the artery wall decreases cardiovascular incidents (for a review, see Green et al. 2017 ²⁶). A degree of caution is warranted, however, as excessive exercise has been shown to have deleterious effects on the cardiovascular system ²⁷, with a reversal in the balance between NO and superoxide production ²⁸.

4 – Mechanism of flow-mediated remodeling

4.1. Shear stress sensing

Many well-documented review articles are available on flow (shear stress)-mediated signal transduction ^{29,30}, including reviews on disturbed flow, which alters large artery walls and induces atherosclerosis ^{31,32}. Nevertheless, the dilation induced by an acute increase in flow involves a rapid response of the ECs, leading to immediate relaxation of the adjacent smooth muscle cells or flow-mediated dilation (FMD). FMD mainly involves the production of NO and a range of COX-derivatives and other hyperpolarizing factors (EDHF) depending on the vessel type, the species, and the disease. Interestingly, FMD can be assessed in patients using a non-invasive technique and it correlates well with endothelial dysfunction and risk factors, although a number of limitations have recently been highlighted ³³.

FMR starts with FMD, which then becomes chronic and changes the arterial wall structure. A first approach to understand the effect of age on FMR is hence to analyze the effect of age on

FMD. The main effect of aging on FMD is a reduction in the production of NO in response to increased shear stress and overexpression of inflammatory factors associated with excessive oxidative stress³⁴. These alterations also characterize the changes in endothelial function observed in cardiovascular and metabolic diseases, which are common, and present as premature vascular (endothelial) aging.

Interestingly, mitochondria are thought to be the biological clock of cells, and ECs are sensitive to oxidative stress of mitochondrial origin^{35, 36}. Furthermore, the mitochondria of ECs undergo hyper-fragmentation in cardiovascular diseases³⁷. Although vascular ECs do not depend on mitochondria for their energy demands³⁸, it has been hypothesized that the mitochondria of ECs play a regulatory rather than an energy-producing role³⁹. As demonstrated in HUVECs exposed to laminar shear stress⁴⁰, shear stress induces a more quiescent and less oxidative phenotype in ECs. Indeed, mitochondrial fusion and fission are sensitive to shear stress in cultured human and bovine ECs⁴¹, and shear stress-dependent Ca²⁺ mobilization in human ECs depends on mitochondria-dependent activation of endoplasmic reticulum channels⁴². Thus, shear stress most probably reduces EC metabolism, with a reduction in mitochondrial ROS production^{40, 43}. It is, therefore, tempting to hypothesize that the reduction in flow sensing and FMD observed with age is associated with increased production of ROS of mitochondrial origin, as discussed below (5.1). Indeed, free radicals produced by the mitochondria in vascular cells have a major role in the peripheral vascular disorders that occur with age^{44, 45}.

4.2. Vascular response to changes in chronic flow

For flow-mediated remodeling (FMR) to occur, there must first be a moderate yet also requisite inflammatory response^{46, 47} as well as a degree of oxidative stress²⁰. As has been shown

in rat mesenteric arteries, the chronic increase in blood flow is followed by an accumulation of perivascular macrophages during the early stage (the first 2 days), as outlined in Figure 2. This has been demonstrated using macrophage inactivation with clodronate, which prevented remodeling of rat mesenteric arteries subjected in vivo to a doubling of blood flow after surgical ligation of the adjacent arteries^{46,48}. Bone marrow cells are also involved in collateral artery growth, as has been documented in the hind limb of mice¹⁰. These findings are in keeping with two studies that have shown that revascularization of the femoral artery after ligation is severely reduced in mice lacking CD4+ cells⁴⁹, CD8+ cells⁵⁰, or natural killer cells⁴⁹, thus highlighting a possible role for T lymphocyte activation in FMR and collateral artery growth. Indeed, in mice lacking T cells (nude mice), FMR of the mesenteric arteries is abrogated⁴⁷. Our analysis of the immune cells surrounding the arteries subjected to increased blood flow revealed an accumulation of T cells two days after the increase in blood flow. These cells expressed markers of Th17 and a high level of angiotensin II type-2 receptor (AT2R)⁴⁷. Interleukin-17 (IL-17) has a protective effect in post-ischemic revascularization in the mouse hindlimb⁵¹, and AT2R activation induces the production of IL-17 by memory T cells⁴⁷. This sequence of events has been confirmed by the observation that FMR does not occur in the absence of AT2R or IL-17 in mice and the finding that the injection of IL-17 into nude mice or mice lacking AT2R restores FMR in mesenteric arteries⁴⁷. The involvement of AT2R in FMR provides a new therapeutic perspective in age-related vascular dysfunction. This could at least in part explain why the AT2R agonist C21 improves ischemic damage in stroke⁵² and has a protective effect in neurodegenerative disorders⁵³. Indeed, we have shown that C21 restored FMR in old mice mesenteric arteries subjected to a chronic increase in blood flow⁴⁷.

The next step after this inflammatory phase comprises activation of matrix metalloproteinases (MMPs), which results in partial digestion of the extracellular matrix in the vascular wall. This process facilitates expansion of the diameter and the ensuing compensatory hypertrophy. MMP9 is most likely involved in FMR, as has been shown in both large^{54,55} and small arteries^{47,56}. An increase in diameter is hence possible and is governed by the artery tone, as demonstrated by Bakker et al.⁴⁶. The main stimulus for the expansion of the diameter is NO that is produced after eNOS activation by flow, as has been shown in both large⁵⁴ and small arteries^{56,57}. The expression level of eNOS is increased and, in parallel, caveolin-1 expression is reduced

so that the NO pathway is more efficient. These changes are activated by IL-17 during the inflammatory phase of the remodeling ⁴⁷. Indeed, IL-17 has a protective effect at low concentrations and it is able to induce eNOS phosphorylation in cultured human ECs ⁵⁸. Thus, IL-17 could also directly activate NO production and thereby increase the arterial diameter. More surprisingly, estrogens are also involved in this phase of the remodeling, as described below. Aside from NO, other vasodilating agents are also recruited in this latter phase, such as CO produced by heme-oxygenase 1 ^{48, 59} and prostanoids synthesized after cyclooxygenase 2 activation ⁶⁰.

Finally, FMR requires an increase in wall thickness in order to normalize tensile stress, which is increased by the expansion of the diameter ^{61, 62}. The increase in diameter leads to excessive wall stress and stretching of smooth muscle cells that activates the local renin-angiotensin system. Consequently, angiotensin II type-1 receptor activation leads to ERK1/2 phosphorylation and smooth muscle hypertrophy ⁶³.

Aside from the chronic increase in flow, which triggers FMR, reversal of the blood flow direction often occurs in collateral vascular segments after occlusion of a feed artery. A study performed on HUVECs cultured in a flow chamber has shown that flow reversal activates a wide panel of pro-arteriogenic signaling pathways ⁶⁴.

4.3. Role of estrogens and estrogen receptor alpha in flow-mediated remodeling

Female sex hormones play a major role in the uteroplacental changes that occur during pregnancy, with major uterine artery outward remodeling associated with trophoblast invasion, hyperplasia, hypertrophy, and modification of the extracellular matrix ². Estrogens activate endometrial secretion of factors that promote vascular smooth muscle cell migration, which is an early step in outward arterial remodeling ⁶⁵. Endogenous 17-beta-estradiol (E2) is essential for increasing endothelium (NO)-mediated dilatation in rat mesenteric arteries ⁶⁶ subjected to chronic high flow, and activation of estrogen receptor alpha (ER α) is essential for FMR in the mesenteric arteries of young female rats ²⁴. Indeed, both ovariectomy of female rats and the absence of ER α in mice prevent FMR in mesenteric arteries ²⁴. Furthermore, endothelial ER α is involved in this FMR, as shown in mice that selectively lack ER α in ECs (Tie2Cre(+))ERf/f mice ²⁴. Furthermore, FMR is controlled by the nuclear activating function 2 (AF2) of ER α . Indeed, ER α -AF2^o mice, which selectively lack the AF2 function, do not develop FMR, whereas mice lacking only the

membrane-associated form of ER α (C451A-ER α mice) respond to the chronic increase in flow with outward remodeling ⁶⁷.

The decline of the amplitude of FMR with age occurs later in female rats than in male rats and is correlated with a progressive decrease in E2 blood levels ¹⁴. This could induce an equivalent decrease in ER α , the expression of which is governed by the level of E2 ¹⁵. While FMR did not occur in young ER α -/- mice, it was reduced by 50% in young ER α +/- mice, thus confirming the importance of gene dosage in FMR. Importantly, increasing E2 blood levels in old female rats could restore FMR to the level observed in young animals ¹⁴. The role of E2 in post-ischemic revascularization has also been demonstrated using a skin flap model in mice ⁶⁸. This effect of E2 on FMR could at least in part explain its protective action in central and peripheral ischemic disorders ⁶⁸⁻⁷⁰. Interestingly, we also observed that E2 lost its capacity to restore FMR in female mice after a 9-month long ovariectomy, thus demonstrating nonreversible loss of the effect of E2 on FMR after prolonged estrogen deprivation ¹⁴. This observation supports the previous works that highlighted the importance of the timing in treating postmenopausal women with E2. Indeed, after a long interruption, the effect of E2 may be more deleterious than beneficial on the cardiovascular system ⁷¹.

5. Flow-mediated remodeling in aging

5.1. Healthy aging

While the probability of an ischemic event increases with age, the capacity of the vascular tree to undergo proper revascularization is reduced. First, FMD is also affected by aging. Ultrasound measurement of FMD in brachial arteries has revealed that both gender and age are independent risk factors for reduced endothelial function ⁷². Thus, the initial stimulus involved in FMR induces less of a dilatory response with age. Indeed, no FMR of first-order mesenteric arteries subjected to a chronic increase in blood flow was noted in healthy male rats that were 12 ⁹ or 24 months ¹² old. Aging also induced a progressive decrease in the collateral number, the diameter, and remodeling after femoral ligation in mice aged 3 to 31 months ⁷³. In this study, aging was accompanied by reduced eNOS signaling, with more nitrosylation and less phosphorylation of

eNOS. However, this age-dependent reduction in collateral growth was not associated with a decrease in T cell or macrophage recruitment ⁷³.

Another study has shown that aging in mice (3 versus 18 months) reduces bone marrow cell recruitment and homing ¹⁰. However, transplantation of young bone marrow into old mice did not result in recovery of blood flow in the leg subjected to ischemia after femoral ligation. A similar result was obtained by another group using an ischemic skin flap model ⁷⁴.

Importantly, aside from the absence of enlargement of the diameter of the arteries subjected to high flow in old male rats, the wall to lumen ratio, which is a key index of the hypertrophy associated with cardiovascular disorders ^{75, 76}, was greater than in young animals. Thus, the consequences are two-fold, with a hypertrophied artery without normalization of shear stress and, above all, no increase in flow, which makes it impossible to compensate for ischemia.

Nevertheless, in relatively healthy aging, despite the absence of an increase in the diameter of arteries subjected to enhanced blood flow, endothelium-mediated dilation in these arteries is enhanced by the chronic increase in flow, as shown in 2-year-old male rats ⁷⁷. In addition, treatment with the vasodilator hydralazine at a dose that does not reduce blood pressure ⁷⁸ restored FMR in the mesenteric arteries of 2-year-old male rats ¹². These observations suggest that the pathways involved in FMR remain efficient, with possibly a shift of the threshold for flow (shear stress) sensitivity of the flow-sensors. This issue remains to be further investigated in order to better define which element could be targeted to improve tissue perfusion in the elderly. One possible explanation could be that the bioavailability of the NO necessary for the increase in diameter in FMR is impaired in aging and that a higher stimulus is needed, as this can be obtained with hydralazine. In old mice subjected to femoral artery ligation, the level of eNOS and phosphorylated eNOS in collateral arteries is lower than in young mice ¹⁰. Furthermore, in old male rats, treatment with an antioxidant drug or with an AT1R blocker can restore FMR of the mesenteric artery to the level obtained in young rats ¹³, which suggests that excessive oxidative stress counteracts the NO pathway involved in FMR. Indeed, oxidative stress increases with age and reduces FMR in aged rats ⁷⁹. Oxidative stress increases with age through several mechanisms such as increased NADPH-oxidase levels and activity ⁷⁹ and through an increase in ROS production by mitochondria ⁸⁰. In old mice treated with the mitochondrial superoxide scavenger MitoTEMPO, the blood flow recovery after femoral occlusion was equivalent to that in young animals ⁸¹. These studies

performed in mice are in keeping with the finding that patients with peripheral artery disease exhibit mitochondrial dysfunction and increased oxidative stress-induced damage^{82, 83}.

Interestingly, E2 has been shown to delay human vascular EC senescence induced by hydrogen peroxide of mitochondrial origin⁸⁴, thus suggesting that estrogens could also prevent the loss of FMR with age, possibly through a reduction in oxidative stress.

5.2. Healthy aging and female hormones

Numerous epidemiological studies suggest that women are better protected than men against cardiovascular diseases and that this effect is lost after menopause⁸⁵. Indeed, the decline in ovarian function is associated with a decrease in NO production¹⁵. FMR has been shown to occur in 12-month-old female but not in male rats of the same age. Compared to females, the NO-dependent pathway was more efficient, and the levels of ERK1/2 phosphorylation, oxidative stress, and vascular contractility were higher in old male rats and in ovariectomized female rats, thus suggesting that excessive oxidative stress opposes NO-dependent enlargement of the arterial diameter. Importantly, in male rats and in ovariectomized female rats, treatment with hydralazine, the antioxidant TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), or candesartan (an inhibitor of the angiotensin II type 1 receptor) normalized FMR and reduced arterial contractility, thus suggesting that estrogens balance oxidative stress and vascular contractility to preserve FMR in mature female rats.^{13, 14}

This role of E2 in FMR through AF2-ER α activation could explain the protective effect of E2 in ischemic disorders, as shown in myocardium, hind limb, brain, and skin^{68, 86-88}. The protective effect of E2 against damage from myocardial ischemia/reperfusion involves the endothelial ER α ⁸⁹, while activation of the non-nuclear ER α pathway decreases the damage from⁹⁰ and the severity of stroke in the mice⁹¹. Using ER α -AF2⁰ and C451A-ER α mice, which have selective inactivation of nuclear (AF2) and membrane ER α , respectively, we have shown that nuclear ER α plays a prominent role in the prevention of the kidney damage induced by angiotensin II-dependent hypertension in old female mice⁹². Interestingly, selective activation of nuclear ER α with estetrol, which is a fetal estrogen, prevents angiotensin II-induced hypertension and promotes FMR⁶⁷. This suggests that pharmacological targeting of the AF2 function of ER α warrants further investigation.

5.3. Flow-mediated remodeling in pathological aging

Unfortunately, aging and risk factors go hand-in-hand, often with fatal consequences. Moreover, hypertension, obesity, and diabetes are increasingly affecting young people. Damage to the vascular wall also accumulates over time, and the frequency of ischemic diseases increases with age. For example, peripheral arterial occlusive disease is mostly observed in the elderly, and its incidence is expected to increase dramatically as the population ages, as well as due to an increase in the prevalence of metabolic syndrome and type 2 diabetes. Thus, there is an urgent need to find new therapeutic strategies to treat and, ideally, to prevent the occurrence and progression of peripheral arterial disease ⁹³

Flow-mediated outward remodeling is severely reduced in spontaneously hypertensive rats (SHRs) ⁹. Indeed, the kinetics of remodeling in very young SHRs is slower than in normotensive WKY rats, and SHRs reach a similar level of FMR in the mesenteric artery after 3 weeks instead of 1 week in WKY rats ⁸. In 10-week-old SHRs, shortly after the onset of hypertension, the damage induced by high intraluminal pressure is minimal. Interestingly, the FMR amplitude decreases after reaching a plateau after 3 weeks, and this is accompanied by wall hypertrophy, hypercontractility, and excessive oxidative stress, which are thought to synergistically abrogate FMR ⁸. In the mesenteric arteries of SHRs, the baseline H₂O₂ and NO levels are higher than in WKY rats, whereas after an increase in chronic flow, only the level of H₂O₂ increased in SHRs, while the level of NO was elevated in WKY rats ⁷. This shift of the response to flow toward the production of H₂O₂ and ROS could participate in the decline in revascularization that occurs with age in association with hypertension ⁹⁴.

Obesity also impairs outward remodeling of collateral arteries. This has been shown in obese mice subjected to femoral occlusion, and it is due to excessive NADPH-oxidase activity and ROS production without a reduction in monocyte mobilization ⁹⁵. Surprisingly, FMR in mesenteric arteries was not reduced in obese rats despite pronounced impairment of NO-dependent relaxation ⁹⁶. However, COX2 inhibition suppressed FMR in old obese rats, thus suggesting that inflammation associated with obesity provides a pathway to compensate for the loss of NO-dependent dilation and to maintain FMR in the mesenteric bed ⁹⁷. This could be specific to the

mesenteric circulation, which is overstimulated in rats with hyperphagia, and this could contribute to the weight gain in these animals.

Flow-mediated outward remodeling is severely reduced in a rat model of type 2 diabetes⁹⁸. In this study, MMP activity was not activated in the arteries subjected to a chronic increase in blood flow, and this is due to the blocking effect of the advanced glycation end-products (AGEs) generated by the hyperglycemia. The AGE-breaker drug ALT-711 reverses this effect in young diabetic rats, resulting in restoration of FMR. However, in old diabetic rats, this drug was ineffective, suggesting that long-term (1 year in the rat) diabetes induces irreversible AGE accumulation⁹⁹ and possibly irreversible tissue damage. In this study, performed in 1-year-old diabetic (ZDF) rats, FMR was not only absent but endothelium-mediated dilation was further decreased, although an antioxidant treatment could partially restore it^{4,99}. In these old diabetic rats, vasoconstrictor prostanoids and oxidative stress both reduced endothelium-mediated relaxation¹⁰⁰.

Finally, unbalanced oxidative stress with excessive superoxide and H₂O₂ production is a common feature of the various risk factors that impair FMR and collateral growth with age. Indeed, H₂O₂ is a potential vasodilating agent that could, at least in part, compensate for the loss of NO. For example, in human coronary arteries from coronary artery disease patients with a median age of 67, flow-mediated dilation depended on the mitochondrial production of hydrogen peroxide instead of nitric oxide¹⁰¹. Although this production of hydrogen peroxide allows dilation to be maintained, it creates an excessively oxidative environment that can lead to extensive tissue damage.¹⁰² Of note, 17beta-estradiol has been shown to delay human vascular EC senescence induced by hydrogen peroxide of mitochondrial origin⁸⁴.

5.4. Therapeutic tools

Exercise has been clearly shown to increase blood flow and to activate FMR, with a positive impact on arterial structure and function²⁶. A sedentary lifestyle is usually associated with other risk factors such as obesity, diabetes, and hypertension. Inactivity per se induces inward remodeling whereas exercise has the opposite effect¹⁰³. Even short-term daily exercise has been

shown to induce remodeling of rat intramural coronary arterioles and to improve coronary blood flow autoregulation¹⁰³. Indeed, well-controlled exercise is recommended for patients with a risk of cardiovascular events²⁶. Unfortunately, there is an increasing trend nowadays in sedentary behavior, with more than one-third of the World population currently below the minimum physical activity requirements needed to remain healthy¹⁰⁴. Thus, alternative solutions are needed, including the use of medications. Aside from specific treatments for diabetes, obesity, and hypertension, which have been described in detail, antioxidant treatments may have the potential to rescue FMR and accelerate revascularization more generally^{5, 95, 99}. As described above (5.1), in healthy aging, increasing blood flow is sufficient to restore FMR, as shown using a non-hypotensive dose of hydralazine in healthy old rats¹². Naturally, healthy aging does not require any treatment, and in aging associated with one or more risk factors, the dilatory machinery is not always efficient. Whereas targeting the renin-angiotensin system with an AT1R blocker and ACE inhibitors is common in cardiovascular and metabolic diseases^{105, 106}, the AT2R is emerging as a potential new therapeutic target.

Importantly, the AT2R is essential for FMR, as shown in mouse mesenteric and skin arteries, and its stimulation restored FMR in 1-year-old male mice⁴⁷. Activation of the AT2R results in opposite and beneficial pharmacological outcomes compared to activation of the AT1R. These include pronounced vasodilator, anti-fibrotic, and anti-inflammatory effects¹⁰⁷. Stimulation of the AT2R could be an alternative to blockade of the renin-angiotensin system in some ischemic diseases that occur with age. Indeed, AT2R stimulation targets only the associated dilatory and anti-inflammatory pathways, without blocking the production of angiotensin II or inhibition of the AT1R, and this is now feasible in light of the development of efficient AT2R agonists¹⁰⁸.

Finally, targeting estrogen receptors is potentially another new therapeutic option, as their numerous functions are now better-described¹⁵. Two pathways lead to immediate vasodilatation with the potential for activation of FMR. First, membrane-associated ER α activates NO-dependent dilation, and selective entities are available to target this function without also stimulating the nuclear functions¹⁰⁹. The G protein-coupled estrogen receptor (GPER) also induces vasodilatation without obvious nuclear effects, and a wide panel of agonists such as natural estrogens, raloxifene, fulvestrant, and a number of phytoestrogens are able to activate this receptor¹¹⁰. Alternatively, targeting the AF2 function of ER α , which is directly involved in FMR⁶⁷, is another option, and pharmacological entities such as estetrol hold considerable promise in this regard¹¹¹.

6. Conclusion

FMR in collateral growth and arteriogenesis is a complex phenomenon that recruits endothelial and smooth muscle cells as well as the immune system. FMR is involved in post-ischemic revascularization, which often occurs in patients with one or several risk factors, including age. Activating FMR in older subjects requires increasing the stimulus on the endothelium by the addition of extra vasodilatation to the initial stimulus (the increase in shear stress). However, as the need for revascularization is greater in older individuals with risk factors, the therapy cannot merely amount to exercise with or without a mild vasodilating drug. Indeed, a key issue is the reversibility of the vascular damage induced over time, and thus promoting prevention and limiting exposure to the risk factors remain the best options in this regard.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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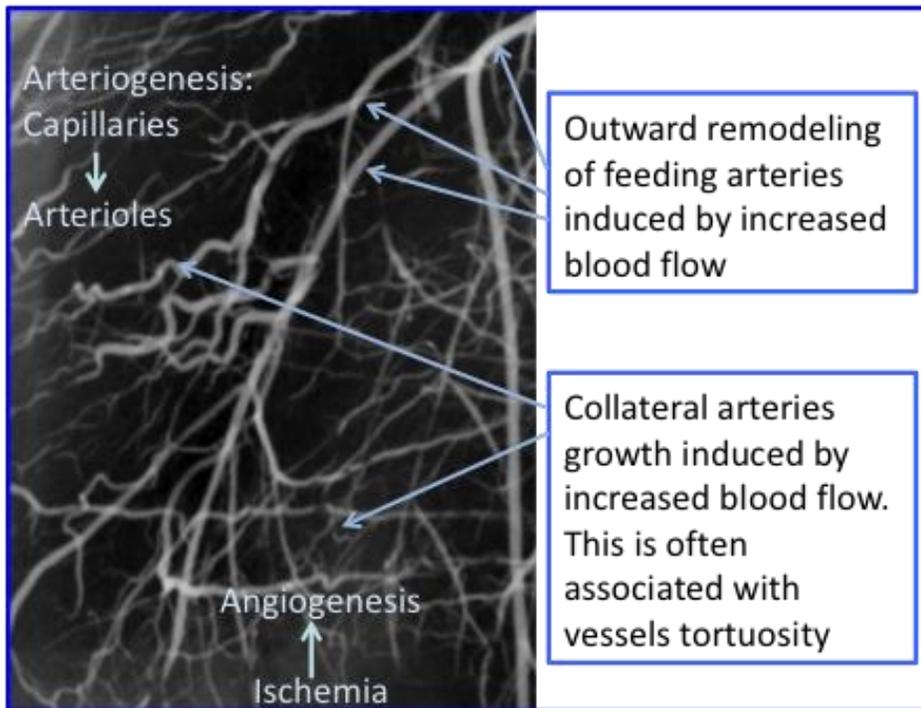


Figure 1: Post-ischemic revascularization. After occlusion of a large artery, small feeding arteries have to increase their flow to compensate for the loss of tissue vascularization. Tissue ischemia triggers angiogenesis, while capillaries undergo arteriogenesis and become arterioles. In parallel, these arterioles become larger in order to carry more blood flow as a result of flow-mediated outward remodeling. The latter also allows feeding arteries to enlarge their diameter.

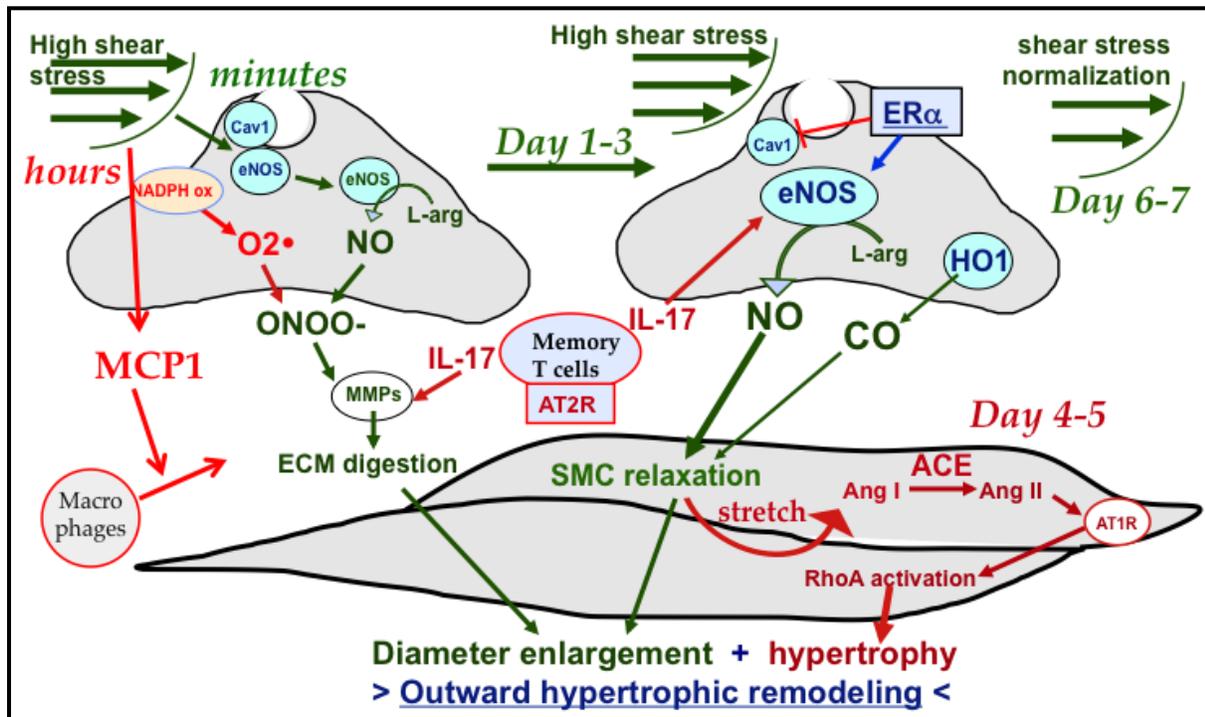


Figure 2: Schematic representation of the vascular response to a chronic increase in blood flow.

In resistance arteries, a chronic increase in blood flow (high shear stress) first activates the NO-pathway and thus the production of NO (within minutes). As the stimulus continues, after several hours a rise in oxidative stress occurs, with the production of superoxide ($O_2\bullet$) and then the production of the monocyte chemo-attractant protein 1 (MCP1), which attracts macrophages to the perivascular space. The association of NO and $O_2\bullet$ produces peroxynitrite ion ($ONOO^-$), which triggers activation of matrix metalloproteinases (MMPs) and extracellular matrix (ECM) digestion. Between days 1 and 3, memory T cells and preferentially Th17 cells produce interleukin-17 (IL-17) upon stimulation of angiotensin II type 2 receptor (AT2R). IL-17 further activates MMPs and also increases eNOS expression. Together with the heme oxygenase-1 expressed during this inflammatory phase, which produces CO, eNOS produces more NO, thereby inducing outward remodeling as an increase in arterial diameter. At this stage, activation of the estrogen receptor alpha ($ER\alpha$) participates in overactivation of the NO pathway through a reduction in caveolin-1 (cav1) expression and an increase in eNOS expression. The enlargement in diameter allows normalization of the shear stress, and the increase in diameter then enhances the tensile stress on the vascular wall, thereby leading to the production of angiotensin II (AngII) by the angiotensin-converting enzyme (ACE) and stimulation of the angiotensin II type 1 receptor (AT1R) and subsequent activation of RhoA, which allows hypertrophy to occur and thus normalization of the tensile stress. Altogether, after 6 or 7 days the vessels become larger and thicker, with normalized shear and tensile stress.

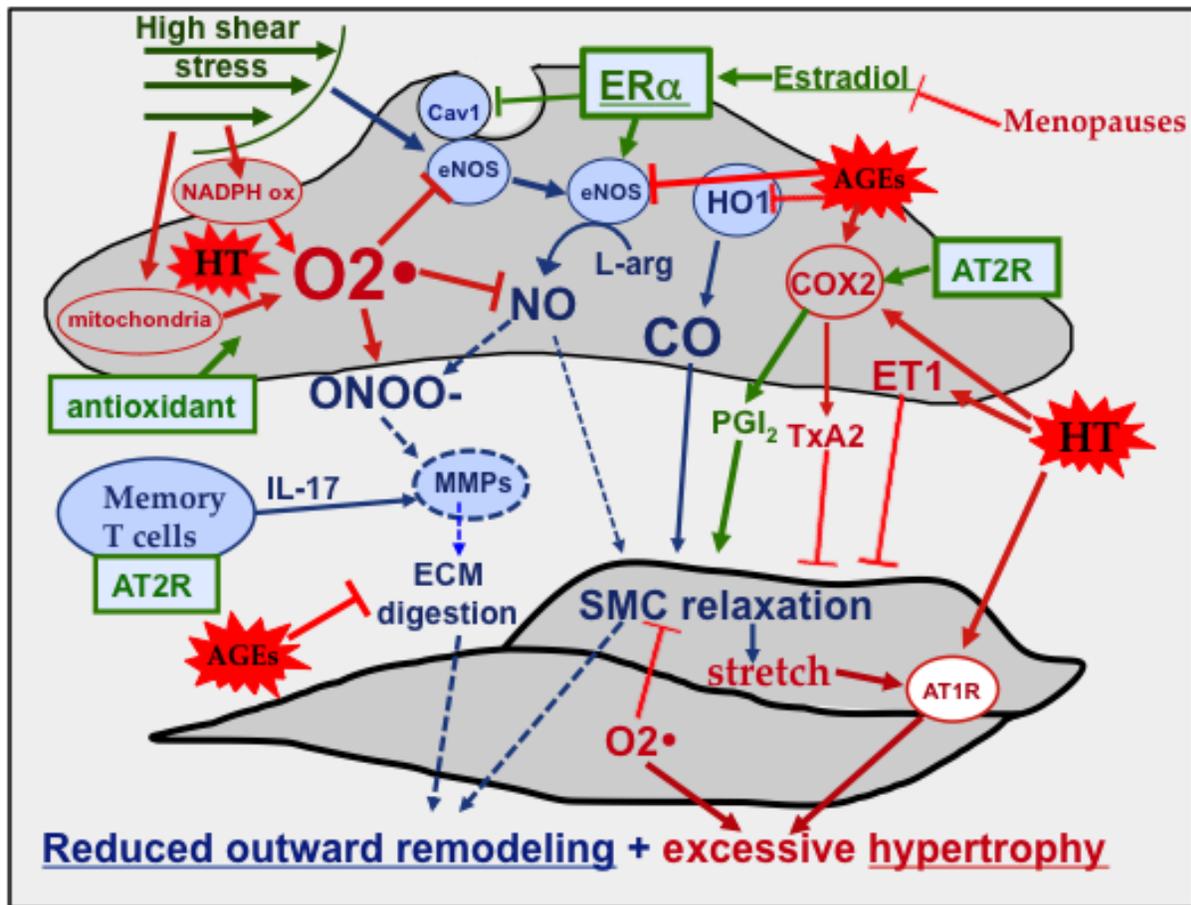


Figure 3: Risk factors (red) that reduce the vascular response to a chronic increase in blood flow. Potential therapeutic strategies are indicated (green).

In blue, the simplified pathway involved in resistance arteries subjected to a chronic increase in blood flow (high shear stress), described in more detail in Figure 2. Hypertension (HT), in addition to age, increases the production of superoxide ($O_2\bullet$), especially of mitochondrial origin. This excessive superoxide production inhibits NO and peroxynitrite ($ONOO^-$) production, matrix metalloproteinase (MMP) activation, and thus extracellular matrix (ECM) digestion. The latter is also inhibited by advanced glycation end products (AGEs) produced in diabetes. Memory T cells, which produce interleukin-17 (IL-17) that leads to activation of MMPs, can be stimulated by agonists of the angiotensin II type 2 receptor (AT2R). AGEs (and also hypertension through activation of superoxide production) also reduce eNOS and HO1 activity, whereas both AGEs and hypertension can activate COX2 expression, which leads to the production of vasoconstricting prostanoids such as thromboxane-A2 (TxA2). Hypertension induces excessive hypertrophy through angiotensin II type 1 receptor (AT1R) activation. **Antioxidant therapy** can reduce most of the deleterious effects of AGEs and hypertension, as long as it is applied early enough. Similarly, activation of the estrogen receptor alpha (ER α) induces NO production, which, together with CO induces expansion of the diameter. Finally, estrogens also have antioxidant properties, but they also have to be used early enough and without any cessation of long-lasting exposure.



Mitochondrial fission and fusion: A dynamic role in aging and potential target for age-related disease



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ABSTRACT

The mitochondria is the major hub to convert energy for cellular processes. Dysregulation of mitochondrial function is one of the classical hallmarks of aging, and mitochondrial interventions have repeatedly been shown to improve outcomes in age-related diseases. Crucial to mitochondrial regulation is the dynamic nature of their network structure. Mitochondria separate and merge using fission and fusion processes in response to changes in energy and stress status. While many mitochondrial processes are already characterized in relation to aging, specific evidence in multicellular organisms causally linking mitochondrial dynamics to the regulation of lifespan is limited. There does exist, however, a large body of evidence connecting mitochondrial dynamics to other aging-related cellular processes and implicates them in a number of human diseases. Here, we discuss the mechanisms of mitochondrial fission and fusion, the current evidence of their role in aging of multicellular organisms, and how these connect to cell cycle regulation, quality control, and transmission of energy status. Finally, we discuss the current evidence implicating these processes in age-related human pathologies, such as neurodegenerative or cardio-metabolic diseases. We suggest that deeper understanding of the regulatory mechanisms within this system and downstream implications could benefit in understanding and intervention of these conditions.

1. The basics of mitochondrial structure

Mitochondria are dynamically interconnected, allowing them to share membranes, solutes, metabolites and proteins, as well as electrochemical gradients. Each mitochondria is organized into two phospholipid bilayers: the outer mitochondrial membrane (OMM), which separates the intermembrane space from the cytosol, and the inner mitochondrial membrane (IMM), which forms cristae into the matrix (Tilokani et al., 2018). While the OMM is mostly permeable to ions and larger molecules, the IMM is less so. Across the IMM an electrochemical gradient is formed, allowing ATP formation through oxidative phosphorylation, heat production through proton leaking, and ROS formation through oxygen reduction (Vafai and Mootha, 2012). Beyond interactions with one another, mitochondria can also connect and exchange materials with other cellular organelles, such as the endoplasmic reticulum (ER) and the lysosomes (Marchi et al., 2014; Soto-Herederó et al., 2017).

2. Fusion and fission proteins and processes

Fusion occurs when two adjacent mitochondria join, while fission separates one mitochondria into two (Sebastián and Zorzano, 2018) (Fig. 1). These two events permanently counterbalance each other; the inactivation of one leads to unopposed action by the other and the subsequent imbalance controls mitochondrial structure (Scorrano, 2013). The molecular mechanisms of these two processes have been previously reviewed in great detail (Tilokani et al., 2018), but here we will briefly describe the conserved proteins (Table 1) and mechanisms that control these processes.

2.1. Fusion

In mammalian cells, fusion is coordinated by the OMM-located mitofusin (MFN) 1, MFN2 and optic atrophy 1 (OPA1), located on the IMM, in separable sequential events (Malka et al., 2005; Song et al., 2009). The mitofusins are dynamin-like GTPases that contain conserved catalytic GTP-binding domains at the N termini, and are anchored to

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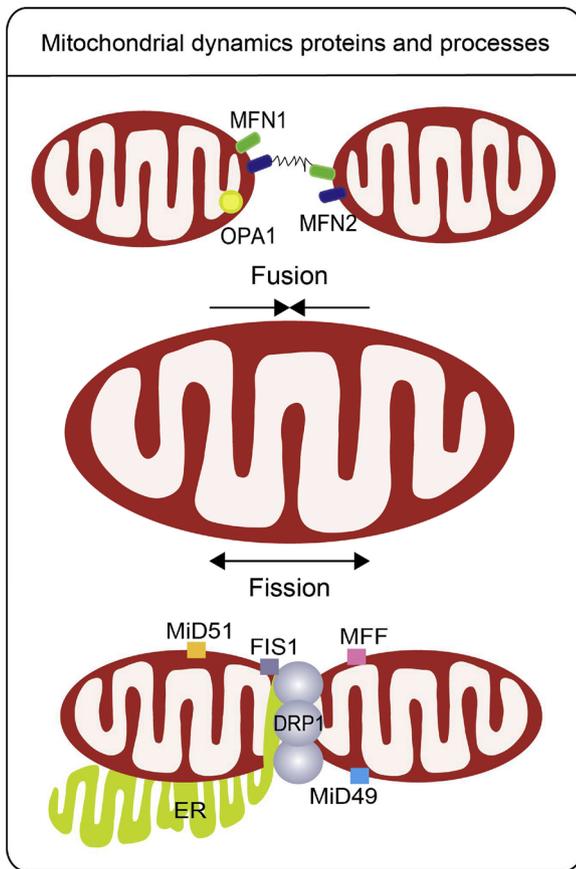


Fig. 1. Simplified model of mitochondrial fusion and fission in human cells. Mitochondrial fusion joins two mitochondria together, while fission separates one into two. Fusion is coordinated on the OMM by the mitofusins (MFN1 and MFN2), and on the IMM by optic atrophy 1 (OPA1). Fission begins when the endoplasmic reticulum (ER) is recruited to the constriction site, marked by mtDNA. Next, multiple OMM-bound proteins (FIS1, MFF, MiD49 and MiD51) recruit DRP1 to the surface of the mitochondria, aiding in ER-mediated constriction.

Table 1
Fusion and fission orthologs.

	Mammalian	C. elegans	D. melanogaster
Fusion proteins	MFN1	FZO-1	FZO
	MFN2		Marf
	OPA1	EAT-3	Opa1
Fission proteins	DRP1	DRP-1	Drp1
	FIS1	FIS-1; FIS-2	Fis
	MFF		Dmel
	MiD49		
	MiD51		
	DNM2		

the OMM by C-terminal transmembrane domains (Wai and Langer, 2016). They each contain two hydrophobic heptad repeats which, during fusion, interact between adjacent mitochondria (Koshiba et al., 2004). The fusion of the OMMs is driven by GTP hydrolysis, which induces a conformational change to bring the opposing membranes in contact with one another (Cao et al., 2017; Qi et al., 2016). MFN1 and MFN2 share approximately 80% similarity (Santel et al., 2003), which is likely the reason that, when overexpressed, each protein is able to rescue the loss of the other to promote fusion (Chen et al., 2003). MFN2 is also present in the ER and controls tethering of the ER to the mitochondria (Basso et al., 2018; de Brito and Scorrano, 2008; Naon et al., 2016), which, as discussed later, aids in the mitochondrial constriction

of the fission process (Cohen et al., 2018).

OPA1 is a dynamin-like GTPase anchored to the IMM by an N-terminal transmembrane domain and is responsible for the fusion of inner mitochondrial membranes (Delettre et al., 2000). Alternative splicing of OPA1 gives rise to long forms (L-OPA1) that can be proteolytically cleaved to short forms (S-OPA1). This cleavage is executed by two IM peptidases: OMA1 and YME1L (Anand et al., 2014). In addition to OPA1, cardiolipin, a mitochondria-specific lipid, is critical to IMM fusion (Ban et al., 2017; Tilokani et al., 2018). The interaction between L-OPA1 and cardiolipin on either side of the membrane tethers the two IMM, which fuse following OPA1-dependent GTP hydrolysis (Liesa et al., 2009). S-OPA1 has been proposed to act as an enhancer of OPA1-CL interaction and fusion (DeVay et al., 2009; Rujiviphat et al., 2009). Mitofusin synthesis is regulated both transcriptionally and with post-transcriptional mechanisms, while their degradation is controlled by ubiquitylation and phosphorylation. OPA1 is regulated post-transcriptionally and post-translationally (Wai and Langer, 2016). Specifically, proteolytic processing plays a large role in regulation of mitochondrial dynamics, as reviewed by (Dietz et al., 2019). A deficiency or loss of fusion proteins leads to mitochondrial fragmentation (Ichishita et al., 2008; Kanazawa et al., 2008).

2.2. Fission

To begin the process of mitochondrial fission, replication of mtDNA in the matrix marks the site for recruitment of the endoplasmic reticulum (ER) (Friedman et al., 2011; Lewis et al., 2016). OMM constriction begins at the mitochondria-ER contact sites, before the oligomerization of DRP1. A number of mitochondrial-bound proteins then aid in the recruitment of DRP1 to the mitochondria, including FIS1, MFF, MiD49, and MiD51, though some of these proteins are known to perform overlapping functions (Loson et al., 2013). Recently, FIS1 was also shown to be involved in lysosomal marking of the mitochondrial fission sites (Wong et al., 2018), thereby further demonstrating inter-organellar communication as an important component of this process and as a point of interest for future study. GTP hydrolysis causes conformational changes, which enhance the ER-mediated constriction (Labrousse et al., 1999; Smirnova et al., 2001).

Interestingly, while FIS1 is not required for DRP1 recruitment to the OMM and therefore fission induction, overexpression of FIS1 can induce mitochondrial fragmentation in the absence of DRP1 (Liesa et al., 2019). Recent evidence demonstrates, in fact, that FIS1 promotes mitochondrial fragmentation both by activating fission and inhibiting fusion (Yu et al., 2019). Human FIS1 blocks fusion machinery by preventing the GTPase activity of MFN1, MFN2, and OPA1 (Yu et al., 2019). This recent work demonstrates how closely connected fission and fusion processes are, and suggests there might be other mechanisms by which one process inactivates the other.

OMM constriction has been well documented, but the mechanism of IMM division is still unclear (Tilokani et al., 2018). Recent work has suggested that IMM constriction is Ca^{2+} -dependent and occurs at mitochondria-ER contact sites, even suggesting a possible role for S-OPA1 in untethering the OMM from the IMM during fission (Chakrabarti et al., 2018; Cho et al., 2017). However, further study is necessary to confirm these results and fully understand the fission process of the IMM.

Mitochondria in cells lacking DRP1 adopt a hypertubular configuration with a highly connected and elongated network in yeast, worms and mammals (Labrousse et al., 1999; Weir et al., 2017). It should be noted that both DRP1 and its receptors also localize to the peroxisomal membranes where they perform membrane fission (Schrader et al., 2016; Waterham et al., 2007). Therefore, studies that ablate DRP1 may also affect cellular processes outside of mitochondrial fission alone. DRP1 can be both activated and inactivated by phosphorylation, depending on the phosphorylated residue, and further regulated by ubiquitylation and SUMOylation (Galvan et al., 2019; Jhun et al., 2018;

van der Bliek et al., 2013). DRP1 phosphorylation can be regulated by a number of factors, including exercise (Moore et al., 2019) and circadian rhythm (Schmitt et al., 2018) though the mechanisms between these processes and the phosphorylation remain unknown.

3. Mitochondrial dynamics in aging

Deterioration in mitochondrial function is one of the nine currently established hallmarks of aging (Lopez-Otin et al., 2013). Over recent years, mild mitochondrial perturbations in various animal models including mice, *Drosophila melanogaster*, and *Caenorhabditis elegans* have been shown to delay aging and age-related functional declines (Cho et al., 2011; Dell'agnello et al., 2007; Dillin et al., 2002; Houtkooper et al., 2013; Lee et al., 2003; Liu et al., 2005). Under these conditions, not only is mitochondrial function affected, but also affect mitochondrial form, seemingly via mitochondrial fission and fusion.

However, the role of mitochondrial dynamics in lifespan regulation is not well elucidated. The yeast field has provided some solid connections, yet there is currently limited evidence causally connecting these two processes in higher organisms. Studies in *Saccharomyces cerevisiae* have shown that fission inhibition increases longevity, while unopposed fission shortens lifespan (Scheckhuber et al., 2007). In line with these observations, studies in *C. elegans* and *D. melanogaster* showed that increased mitochondrial fusion is associated with increased longevity (Chaudhari and Kipreos, 2017; Rana et al., 2017), and in worms, fragmentation of the mitochondrial network correlates with age (Houtkooper et al., 2013; Jiang et al., 2015; Regmi et al., 2014) (Table 2). However, yeast and worms yield opposite effects on longevity upon simultaneous ablation of both fission and fusion processes. In yeast, doing so shortens replicative lifespan (Bernhardt et al., 2015) while in worms a frozen mitochondrial network promotes longevity (Weir et al., 2017). Though these differences between unicellular and multicellular organisms are interesting, and certainly warrant future study, the subsections presented below focus on complex, multicellular animal models.

A number of studies have shown that mitochondrial dynamics are required for lifespan extension in distinct long-lived conditions, including TORC1-mediated longevity, AMPK-mediated longevity, and upon dietary restriction (Burkewitz et al., 2015; Weir et al., 2017; Zhang et al., 2019) (Table 2). Similarly, in mice, caloric restriction (CR) caused increased mitochondrial length and width in muscle fibers, and this effect was dependent on the mitochondrial biogenesis transcriptional coactivator, PGC-1 α (Finley et al., 2012). However, more study is necessary to understand these connections, as different longevity regimens cause different cellular responses involving distinct molecular mechanisms. While AMPK-mediated longevity requires both fission and fusion (Weir et al., 2017), longevity induced via TORC1 specifically requires mitochondrial fusion (Zhang et al., 2019). Considering this context dependence, it will be necessary to unravel the mechanisms further to understand how these processes are required for specific lifespan extension interventions.

While much remains to be studied to causally connect mitochondrial dynamics with lifespan per se, there already exists a strong body of evidence in multicellular animal organisms tying mitochondrial dynamics to specific cellular and molecular hallmarks of aging. We discuss below how mitochondrial dynamics specifically affect cell viability, senescence, mitochondrial health, bioenergetics, quality control and intracellular signaling. Each of these processes plays an important role in aging, tying even more tightly together the connections between mitochondrial form and function with longevity.

3.1. Mitochondrial function and energy states

Different mitochondrial network states are associated with specific nutrient states. A nutrient-rich environment tends to accompany a fragmented mitochondrial network, whereas starvation leads to

elongated and fused mitochondria (Liesa and Shirihai, 2013; Rossignol et al., 2004; Tondera et al., 2009). While these effects are not universal, and do not lead to unopposed fission or fusion, imbalance of the system has led to these observations. The network exhibits a high level of plasticity, allowing rapid rescue if nutrient status changes (Schrepfer and Scorrano, 2016). Mitochondrial fusion tends to lead to an increase in bioenergetics efficiency, maintaining ATP production even upon limited nutrients and therefore allowing the cell to maintain viability (Gomes et al., 2011; Rambold et al., 2011). Meanwhile, mitochondrial fragmentation is associated with increased oxidative stress, mitochondrial depolarization, and reduced ATP production (Jheng et al., 2012). The decreased bioenergetics efficiency may serve as a protective mechanism from the detrimental effects associated with nutrient overload (Liesa and Shirihai, 2013).

These metabolic inputs regulate the proteins and processes of fission and fusion. DRP1 particularly seems to play a large role. Under starvation conditions, PKA phosphorylates DRP1, thereby blunting the fission process (Gomes et al., 2011; Rambold et al., 2011). Conversely, excess of glucose is also dependent on DRP1 to fragment mitochondria and increase levels of ROS (Yu et al., 2006), suggesting a potential role for DRP1 in treatment of metabolic diseases, as discussed below.

Upon *MFN2* deletion, mammalian cells have decreased oxygen consumption, increased proton leak, reduced mitochondrial membrane potential, increased production of ROS, thereby leading to reduced glucose, pyruvate and fatty acid oxidation (Bach et al., 2003; Mourier et al., 2015; Sebastian et al., 2012). Interestingly, one study in this cell model demonstrated that insufficient coenzyme Q levels could explain the deficient respiratory chain activity (Mourier et al., 2015). Similarly, knockdown of *OPA1* reduces mitochondrial membrane potential and increases proton leak, leading to overall reduction in basal, ATP-coupled, and maximal respiration (Buck et al., 2016; Chen et al., 2005; Zhang et al., 2011). Contrary to *Mfn2* deletion, an increase in mitochondrial respiration and ROS production is observed in POMC neurons with ablated *Mfn1* (Ramirez et al., 2017). However, the effect was not conserved in mice missing *Opa1* in POMC neurons (Ramirez et al., 2017), suggesting the effect is specific to *Mfn1*, not mitochondrial fusion.

3.2. Cell viability and senescence

Cell proliferation requires a great deal of energy, and these energy states seem, at least in part, to be regulated by the mitochondrial network. Mitochondria elongate in the G1 and S phases, creating an energy-conservative state to provide enough ATP for cell replication (Mitra et al., 2009; Schieke et al., 2008; Schrepfer and Scorrano, 2016). Entry into S phase can be induced by *Drp1* knockdown via increased cyclin E expression (Mitra et al., 2009), demonstrating the balance between mitochondrial fission and fusion, even in the context of cell cycle regulation.

In the G2/M phases, mitochondria tend to fragment, presumably to ensure equal distribution of mitochondria between daughter cells (Mitra et al., 2009; Taguchi et al., 2007). Knockdown of *FIS1* causes replicative stress and G2/M arrest (Lee et al., 2014; Qian et al., 2012; Salazar-Roa and Malumbres, 2017). Regulation of mitochondrial dynamics and cell cycling has been reviewed previously in more depth (Horbay and Bilyy, 2016; Salazar-Roa and Malumbres, 2017).

Cellular senescence, whereby cells cease division and enter a quiescent state, characterized by the secretion of inflammatory signaling factors, is a well-described hallmark of aging (Lopez-Otin et al., 2013). Senescent cells have a decreased capacity for mitochondrial biogenesis and ATP synthesis (Liesa and Shirihai, 2013). Aged organisms and tissues tend to accumulate senescent cells, and their clearance increases lifespan (Baker et al., 2016, 2011; Gorgoulis et al., 2019; Xu et al., 2018). Senescent cells have reduced *FIS1* and *DRP1* expression, with slightly increased MFN protein levels (Gomes et al., 2011; Lee et al., 2007; Yoon et al., 2006). Knockdown of *FIS1* in human cells also

Table 2
Summary of mitochondrial morphological alterations in aging and aging-related diseases. Specific examples are discussed more elaborately in the main text.

Disease/lifespan phenotypes	Mitochondrial network	Mitochondrial dynamics proteins	References
Neurodegenerative disease			
CMT2A: peripheral neuropathy	Fragmented	MFN2 mutation in humans	(Vance, 2009; Zuchner et al., 2004)
DOA: loss of RGCs	Fragmented	OPA1 mutation in humans	(Votruba et al., 1998)
PD: dopaminergic neurodegeneration	Fragmented	DRP1-mediated excessive fragmentation	(Dagda et al., 2009; Lutz et al., 2009)
AD: ubiquitous neurodegeneration	Fragmented	DRP1 activation through S-nitrosylation	(Cho et al., 2009)
Cardiac dysfunction			
HD: neurodegeneration in basal ganglia and cerebral cortex	Fragmented	Elevated GTPase DRP1 activity	(Shirendeb et al., 2011)
Cardiomyopathy and heart failure in mice	Fragmented	<i>Mfn1</i> / <i>Mfn2</i> knockout in mouse heart	(Jin et al., 2011)
Cardiomyopathy in mice	Fragmented	Heterozygous OPA1 mice	(Chen et al., 2012)
Dilated cardiomyopathy and cardiomyocyte necrosis in mice	Mitochondrial enlargement	<i>Drp1</i> knockout in mouse heart	(Song et al., 2015)
Cardiac hypertrophy in mice	Heterogenous size	<i>Mfn1</i> / <i>Mfn2</i> / <i>Drp1</i> triple knockout in mouse heart	(Song et al., 2017)
Metabolic dysfunction			
Obese Zucker rats and obese humans	Fragmented	Reduced <i>Mfn2</i> expression	(Bach et al., 2003)
Glucose intolerance and insulin resistance in both liver and muscle in mice	Fragmented	<i>Mfn2</i> deletion in mice liver	(Sebastian et al., 2012)
Obese mice	Fragmented	Increased protein level of DRP1	(Jheng et al., 2012)
Lifespan extension of wild type <i>C. elegans</i>	Tubular mitochondria	<i>drp-1</i> / <i>fzo-1</i> double mutations	(Weir et al., 2017)
Longevity model of insulin/IGF signaling inhibition in <i>C. elegans</i> , <i>daf-2(e1370)</i>	Increased fusion compared to wild type	Increased protein level of EAT-3	(Chaudhari and Kipreos, 2017)
Lifespan extension of <i>daf-2(e1370)</i>	Giant globular mitochondria	<i>drp-1</i> mutation	(Yang et al., 2011)
Dietary restriction model in <i>C. elegans</i> , <i>eat-2(ad1116)</i>	Increased fusion compared to wild type	unknown	(Chaudhari and Kipreos, 2017; Weir et al., 2017)
Longevity model of LET-363/TOR inhibition in <i>C. elegans</i> , <i>let-363</i> RNAi, null mutations in <i>raga-1</i> (Raga homolog) or <i>rsk-1</i> (S6K homolog)	Increased fusion compared to wild type	unknown	(Chaudhari and Kipreos, 2017; Zhang et al., 2019)
Lifespan extension in <i>D. melanogaster</i>	Increased fission in midlife of animals	<i>Drp1</i> or <i>p62</i> overexpression in midlife of animals	(Aparicio et al., 2019; Rana et al., 2017)

triggers premature senescence-associated phenotypes, such as lysosomal accumulation, which can be reversed by abrogation of mitochondrial dynamics through additional knockdown of OPA1 (Lee et al., 2007). Additionally, while senescence is related to hyper-tubular mitochondrial networks, apoptosis is associated with mitochondrial fragmentation (Frank et al., 2001). Altogether, these data suggest that an individual cell's life cycles are marked by distinct mitochondrial network structures, responding in large part to balance specific energy states. However, there is limited recent evidence tying together the role of mitochondrial dynamics and cellular senescence. This would be an interesting topic for future research as it connects two well-established hallmarks of aging.

3.3. Mitochondrial quality control mechanisms

3.3.1. Mitochondrial proteostasis

Aging is accompanied by a gradual loss of proteostasis and interventions that maintain proteostasis, such as by increasing molecular chaperone expression or promoting protein turnover, have been implicated in health and longevity in animals (Jensen and Jasper, 2014). For example, the mitochondrial unfolded protein response (UPR^{mt}) is a mitochondria-to-nucleus retrograde signaling cascade that limits protein import and induces expression of chaperone and protease genes to maintain mitochondrial protein homeostasis (Haynes and Ron, 2010). Studies in *C. elegans* have shown that moderate mitochondrial dysfunction resulting from inhibition of mitochondrial translation or mitochondrial electron transport chain activate the UPR^{mt} to promote longevity (Durieux et al., 2011; Houtkooper et al., 2013).

The instrumental role of mitochondrial dynamics in mitochondrial proteostasis lies in the concept of fusion-mediated content mixing and fission-mediated separation. Fusion allows the exchange of mitochondrial contents between adjacent mitochondria which facilitates functional complementation in the face of mitochondrial deficits (Liu et al., 2009). In contrast, fission allows an equal distribution of the mitochondrial proteome and mtDNA in two daughter mitochondria during mitochondrial biogenesis (Mishra and Chan, 2014). Fission also functions by segregating dysfunctional and superfluous mitochondria for degradation through mitochondrial autophagy (mitophagy, discussed below). A direct link between the mitochondrial network and mitochondrial proteome homeostasis has been suggested by studies in *C. elegans*, where genetically blocking fission or fusion evokes protein misfolding stress, thereby activating the UPR^{mt} (Zhang et al., 2018). Moreover, loss of mitochondrial protein homeostasis coincides with dysregulation of mitochondrial dynamics and abnormal mitochondrial morphology during aging (Houtkooper et al., 2013; Leduc-Gaudet et al., 2015; Moehle et al., 2019; Sebastian et al., 2016). It will be interesting in the future to test if rebalancing fission and fusion will revert the decline of mitochondrial protein homeostasis so as to benefit longevity and health in aged animals.

3.3.2. Mitochondrial DNA integrity

Loss of mtDNA integrity and stability is a major cause of mitochondrial dysfunction and the accumulation of mtDNA mutations with age has been implicated in cellular dysfunction (Kujoth et al., 2005; Linnane et al., 1989; Trifunovic et al., 2004). Knock-in mice expressing a proofreading-deficient form of polymerase POLG γ , the so-called 'mutator mice', significantly accumulate mtDNA mutations in various tissues and exhibit premature aging phenotypes such as hair loss, graying, sarcopenia, and deafness (Kujoth et al., 2005).

Mitochondrial fusion is important for maintaining mtDNA levels and mtDNA fidelity (Chen et al., 2010; Papanicolaou et al., 2012a). Patients with OPA1 mutations harbor mtDNA deletions in their skeletal muscle, suggesting an association between fusion and mtDNA instability (Amati-Bonneau et al., 2008; Hudson et al., 2008). In addition, reducing mitochondrial fusion by conditionally ablating mitofusins in mouse skeletal muscle or heart results in profound reductions in mtDNA

content, great accumulations of mtDNA mutations, and development of mitochondrial myopathy in both muscle and heart (Chen et al., 2010; Papanicolaou et al., 2012a). Moreover, fusion also confers high tolerance to mtDNA lesions in cells through content mixing, thereby suppressing expression of mitochondrial dysfunction-related disease phenotypes in mice (Nakada et al., 2001; Ono et al., 2001). Conversely, blocking fission in mouse embryonic fibroblasts does not have a significant effect on mtDNA levels (Ishihara et al., 2009). Overall, these results demonstrate the beneficial effects of fusion in safeguarding mtDNA integrity, thereby highlighting fusion as a potential therapeutic target for aging and mtDNA-related diseases (Chen et al., 2010; Nakada et al., 2009).

3.3.3. Mitochondria-derived vesicles

Mitochondria-derived vesicles (MDVs) recently emerged as a mitochondrial quality control pathway that acts to remove oxidized proteins and lipids in mitochondria. This pathway provides a fine-tuned mitochondrial repair system before accumulation of too much damage, thereby preventing complete elimination of mitochondria by mitophagy. Although investigation of the factors constituting MDVs transport system is just begun, it seems that some essential components of the mitophagy process also participate in lysosome-targeted MDV formation (Sugiura et al., 2014). For example, mitochondrial ROS induced by the uncoupler CCCP and the complex III inhibitor antimycin A in immortalized human cells evokes formation of MDVs in a PINK1- and Parkin-dependent manner (McLelland et al., 2014). Mechanistically, instead of relying on the established DRP1-involved fission machinery, new mediators, so far undescribed, sever MDVs from mitochondria (McLelland et al., 2014; Neuspiel et al., 2008; Soubannier et al., 2012a). This conclusion is supported by ultrastructural analysis: the size of MDVs is between 70 nm–150 nm, whereas the diameter of the yeast mitochondrial dynamin (Dnm1) spirals that drive mitochondrial constrictions is restricted to 100 nm (Ingerman et al., 2005; Soubannier et al., 2012a, b). Therefore, DRP1-formed spirals are too large to constrict a MDV neck, meaning another mediator is required.

Given the connection between mitophagy and MDVs, dysfunction of MDVs is speculated to trigger premature mitophagy, which consequently impairs the hierarchical surveillance network of mitochondrial quality control mechanisms, encompassing the UPR^{mt}, mitochondrial fission and fusion, MDVs, and mitophagy (Sugiura et al., 2014). As such, deficiency in MDVs may contribute to aggregation of global cellular damage and dysfunctional mitochondria during aging (Sugiura et al., 2014). Understanding the molecular mechanisms required for formation and trafficking of MDVs will foster our understanding about how cargoes are specifically incorporated into MDVs, the role of MDVs in mitochondrial quality control, and also how defects in MDVs pathways are implicated in human diseases.

3.3.4. Mitophagy

Mitophagy is a specific form of autophagy that exclusively eliminates damaged and superfluous mitochondria in cells (Ding and Yin, 2012). A functional decline in mitophagy results in the accumulation of dysfunctional mitochondria, a shared hallmark of aging and aging-related pathologies (Lopez-Otin et al., 2013; Sun et al., 2016). Many mechanistic aspects of mitophagy were resolved in yeast (for a comprehensive overview we refer the reader to (Furukawa et al., 2019; Kanki et al., 2015)), but the links between mitophagy and animal aging are less clear.

Several mitophagy signaling cascades are differentially activated in response to various stimuli under distinct cellular contexts in mammalian cells (Palikaras et al., 2017; Wei et al., 2015). Depending on whether ubiquitin (Ub) is required for the recognition of cargoes for degradation, mitophagy pathways can be classified into ubiquitin-dependent and mitochondrial receptor-mediated mechanisms (Khaminets et al., 2016; Pickles et al., 2018) (Fig. 2). Much of what is known about ubiquitin-dependent mitophagy involves PINK1 and Parkin that, when mutated, can cause familial Parkinson's diseases (Kitada et al., 1998; Valente et al., 2004). Upon stresses such as mitochondrial depolarization or mitochondrial proteotoxicity, PINK1 accumulates on the mitochondrial surface and recruits ubiquitin E3 ligase Parkin, which ubiquitinates outer mitochondrial membrane (OMM) proteins to facilitate mitochondria removal (Harper et al., 2018; Matsuda et al., 2010; Ordureau et al., 2014). Mitochondrial receptor-mediated mitophagy occurs in response to specific stresses such as hypoxia or during erythropoiesis. Upon activation, the OMM proteins, such as BCL-2-like protein 13 (BCL2L13), NIX (also called BCL2/adenovirus E1B-interacting protein 3-like, BNIP3L), BNIP3, and FUNDC1 (FUN14 domain-containing protein 1), serve as mitophagy receptors to interact with autophagosomes through their LC3-interacting region (LIR) motifs and promote mitochondrial degradation (Gatica et al., 2018; Hamacher-Brady et al., 2007; Liu et al., 2012; Murakawa et al., 2015; Quinsay et al., 2010; Rogov et al., 2017).

Mitochondrial dynamics is a common target of various mitophagy pathways to initiate the degradation process. Upon mitochondrial depolarization, MFN1 and MFN2 are ubiquitinated by Parkin, which promotes their degradation, thereby abolishing fusion, promoting mitochondrial fragmentation, and facilitating mitophagy (Gegg et al., 2010; Ziviani et al., 2010). In addition, in most cancer cells where Parkin is not expressed, the E3 ubiquitin-protein ligase ARIH1 ubiquitinates MFN2 and promotes its degradation through the proteasome upon CCCP-induced mitophagy (Villa et al., 2017). Moreover, MFN2 is phosphorylated by PINK1 to recruit Parkin to impaired mitochondria upon depolarization-induced mitophagy in mouse cardiac myocytes (Chen and Dorn, 2013). PINK1 also upregulates DRP1 activity in mitophagy by preventing its phosphorylation from protein kinase A (PKA), which promotes segregation of damaged mitochondria for

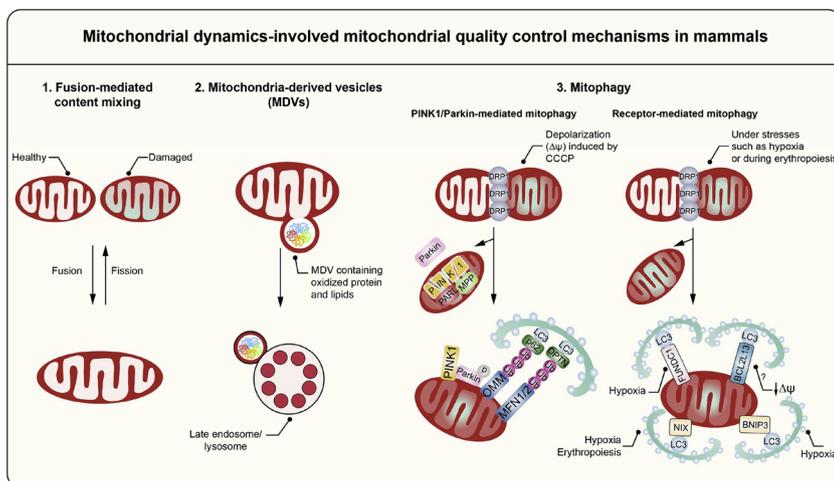


Fig. 2. Mitochondrial quality control mechanisms involving mitochondrial dynamics. Mitochondria harbor a network of surveillance mechanisms encompassing (1) fusion-mediated function complementation, (2) mitochondria-derived vesicles, and (3) mitophagy processes to protect mitochondrial homeostasis from increasing degrees of damage. Specifically, fusion-mediated functional complementation and mitochondria-derived vesicles constitute the first line of defense against mild mitochondrial impairment. Upon an increase in the impairment within the mitochondria, damaged compartments are segregated from the mitochondrial network through fission and subsequently undergo mitophagy. According to the type of stress, different molecular machineries are responsible for mitophagy. For instance, PINK1/Parkin mediates mitophagy in the face of mitochondrial depolarization while, in mitophagy induced by hypoxia or during erythropoiesis, the connection of damaged mitochondria to nascent autophagosomes is built by mitochondrial receptors such as NIX, BNIP3, FUNDC1, and BCL2L13 through interaction with LC3.

degradation (Pryde et al., 2016). Additionally, under the stress of hypoxia in HeLa cells, FUNDC1 activated DRP1 in mitophagy (Chen et al., 2016; Liu et al., 2012). Collectively, these data show that mitochondrial fragmentation is necessary in mitophagy pathways.

Conventionally, the role of mitochondrial division in mitophagy is thought to fit long and tubular mitochondria into nascent autophagosomes (Gomes et al., 2011; Kageyama et al., 2014; Rambold et al., 2011; Tanaka et al., 2010). Indeed, a number of studies have provided evidence in support of the necessity of fission in the induction of mitophagy (Gomes et al., 2011; Ikeda et al., 2015; Kageyama et al., 2014; Kanki et al., 2009; Rambold et al., 2011; Tanaka et al., 2010; Twig et al., 2008). However, some forms of mitophagy, for instance when induced by hypoxia, occur independent of mitochondrial division (Yamashita et al., 2016). In addition, studies in yeast also found that Dnm1 is dispensable for mitophagy (Bernhardt et al., 2015; Kanki et al., 2009; Mendl et al., 2011; Yamashita et al., 2016). Therefore, it is still controversial whether fragmentation mediated by the canonical core components of mitochondrial fission machinery is a prerequisite for mitophagy.

It should be noted that the current understanding of the role of mitochondrial division in mitophagy described above is based predominately on experimental evidence obtained after massive induction of mitophagy, such as using mitochondrial membrane potential uncouplers. This causes severe impairments of mitochondrial and cellular functions, which does not represent situations under physiological conditions. One study instead activated PINK1-Parkin-mediated mitophagy by expressing the mitochondrial-localized mutant ornithine transcarbamylase (Δ OTC) in mammalian cells to better represent physiological conditions (Burman et al., 2017). They reported that rather than being required for mitophagy, DRP1 selectively segregated mitochondrial subdomains that contain accumulated misfolded protein aggregates for elimination through mitophagy (Burman et al., 2017). Furthermore, a recent study revealed a novel function of DRP1 in affecting mitochondrial membrane potential (Cho et al., 2019). Specifically, under normal conditions, DRP1 can transiently reduce mitochondrial membrane potential at division sites in mammalian cells (Cho et al., 2019). This reduction in mitochondrial membrane potential was rapidly restored in healthy mitochondria, whereas damaged mitochondria failed to do so and therefore underwent mitophagy (Cho et al., 2019). This is proposed as a routine surveillance mechanism and negative selection pressure to maintain mitochondrial quality (Cho et al., 2019). It is necessary in future studies to determine the functions of DRP1 and DRP1-mediated fission in mitophagy in different physiological contexts to gain a comprehensive understanding of the significance of mitochondrial fission in mitochondrial homeostasis.

Mitophagy maintains mitochondrial homeostasis and its decline

with age contributes to an accumulation of dysfunctional mitochondria, a cause of numerous diseases of old age (Lopez-Otin et al., 2013; Sun et al., 2016; Wallace, 2005). Interventions that boost mitophagy benefit health and longevity in multiple model organisms ranging from yeasts to mice (Aparicio et al., 2019; Palikaras et al., 2015; Rana et al., 2017; Ryu et al., 2016; Schiavi et al., 2015). For example, studies in *C. elegans* reveal an important role of mitophagy in longevity assurance (Palikaras et al., 2015; Schiavi et al., 2015). Inhibiting mitophagy through loss of the mitophagy regulators *dct-1* (NIX/BNIP3L homolog) or *pink-1* (PINK1 homolog) leads to an accumulation of dysfunctional mitochondria and compromises animals' resistance to various types of stress such as oxidative stress, heat stress, and starvation (Palikaras et al., 2015). Moreover, in long-lived *C. elegans* such as the insulin/IGF1 receptor mutant *daf-2* and animals with moderate mitochondrial dysfunction, induction of mitophagy through *dct-1* or *pink-1* is key to their longevity assurance (Palikaras et al., 2015; Schiavi et al., 2015). Studies in *D. melanogaster* provide further support of a direct role of mitophagy in lifespan determination (Aparicio et al., 2019; Rana et al., 2017). Upregulation of an autophagy receptor p62 (also known as Sequestosome 1) in middle-aged flies promotes mitochondrial fission and mitophagy to prolong lifespan. In addition, *Drp1* overexpression in aging flies reverses age-related mitochondrial enlargement, restores mitochondrial respiratory function, and facilitates mitophagy, so as to delay age-onset pathology and prolong lifespan (Rana et al., 2017). Moreover, pharmacological activation of mitophagy through dietary supplementation of urolithin A also prolongs *C. elegans* lifespan (Ryu et al., 2016). Urolithin A treatment in *C. elegans* induces mitochondrial fragmentation and autophagy-dependent mitochondrial removal (Ryu et al., 2016). In aged mice, urolithin A stimulates autophagy in gastrocnemius muscle and prevents age-related decline of muscle function (Ryu et al., 2016). In summary, these findings suggest that targeting mitochondrial fission-mitophagy axis provides promising therapeutic choices to benefit organismal health and lifespan.

3.4. Bioenergetics and metabolic signaling pathways

Nutrients are vital for life. Therefore, it is not surprising that the prime signaling pathways that function in sensing, absorbing, and consuming nutrients also modulate the rate of aging. These include the insulin/insulin-like growth factor 1 (IIS) signaling pathway, mammalian target of rapamycin (mTOR), and AMP-activated kinase (AMPK) pathways (Houtkooper et al., 2010; Templeman and Murphy, 2018).

An increasing body of evidence suggests the importance of mitochondrial morphology in these energy-sensing signaling pathways to influence metabolism, health, and longevity (Fig. 3). Studies in *C. elegans* show that genetically suppressing the IIS receptor DAF-2 increases

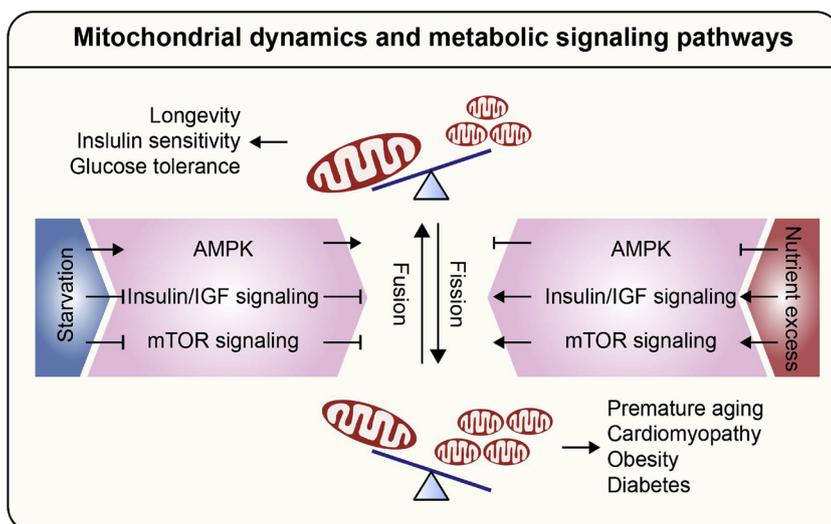


Fig. 3. The role of mitochondrial fission and fusion in metabolic signaling pathways. Generalized and simplified summary of the main pathways linking nutritional state with mitochondrial dynamics and their ultimate phenotypic outcome. AMPK, insulin/IGF, and mTOR nutrient signaling pathways constitute a core network in mammalian cells to coordinate metabolism and regulate lifespan. Depending on the availability of nutrients, these signaling pathways are differentially switched on or off, corresponding to specific mitochondrial morphologies. For example, under the condition of starvation, AMPK is activated, while insulin/IGF and mTOR signaling are suppressed. This change typically favors mitochondrial fusion, which is associated with major beneficial effects such as prolonged lifespan, improved insulin sensitivity, and enhanced glucose tolerance. In contrast, nutrient excess activates insulin/IGF and mTOR signaling but represses AMPK. This leads to an activation of mitochondrial fission, which is associated with a number of human pathologies such as aging, cardiomyopathy, obesity, and diabetes. It should be noted that these responses are not uniform and are highly context-dependent.

the level of mitochondrial fusion by preventing degradation of EAT-3 (OPA1 homolog in *C. elegans*), and the fused mitochondrial network is required for DAF-2 inhibition-mediated longevity (Chaudhari and Kipreos, 2017). Conversely, promoting mitochondrial fusion by depleting the fission gene *drp-1* amplifies the effect of reduced insulin signaling on longevity (Yang et al., 2011). Inhibiting LET-363/TOR in *C. elegans* also engenders a fused mitochondrial network, which is necessary for its beneficial effects on longevity (Chaudhari and Kipreos, 2017). Furthermore, inhibiting mTOR abrogates translation of its target gene mitochondrial fission process 1 (MTFP1) in mouse embryonic fibroblasts, thereby causing mitochondrial hyperfusion and protecting cells from apoptosis (Morita et al., 2017). In contrast to the pro-fission effect of insulin signaling, as described above, studies in cultured rat cardiomyocytes reported that a 3 h treatment of insulin upregulates OPA1 at the protein level and leads to an interconnected mitochondrial network partially through the Akt/PKB-mTOR signaling pathway (Parra et al., 2014). A similar disconnect involving bimodal effects on mitochondrial dynamics appears to happen upon CR. CR is by far the most effective and non-invasive way to improve health and decelerate aging in most organisms through an AMPK-dependent mechanism (Fontana et al., 2010). An increase in mitochondrial size has been reported in skeletal muscle from caloric restricted mice, which may contribute to CR-mediated improvement of mitochondrial function (Finley et al., 2012). Moreover, CR and constitutively active AAK2 (AMPK α 2 homolog) was shown to prolong lifespan in *C. elegans* by maintaining mitochondrial network connectivity (Weir et al., 2017). Overexpressing of constitutively active AMPK in human endothelial cells also promoted mitochondrial fusion by lowering the protein level of DRP1 (Wang et al., 2017). In contrast, upon acute mitochondrial stress such as inhibition of mitochondrial electron transport chain through treatment of rotenone or antimycin A, AMPK mediated mitochondrial fragmentation in cultured human cells (Toyama et al., 2016). Short-term pharmacological (1 h) activation of AMPK was also sufficient for mitochondrial fragmentation in cultured human cells (Toyama et al., 2016). The apparent discrepancy regarding the effect of insulin and AMPK signaling on mitochondrial network might be, at least partially, explained by the varied type (acute vs mild) and duration (long-term vs short-term) of stress used in cell lines or animal models. Together, these results highlight that the adaptation of the mitochondrial network is required by these nutrient-sensing pathways to exert beneficial effects on longevity. Future studies are warranted to determine the mechanism by which mitochondrial morphology engages in lifespan regulation upon activation of various nutrient-sensing signaling pathways. In this sense, it will be interesting to evaluate whether the outputs of fission and fusion such as mitochondrial respiration, mitophagy, and ROS production are associated with longevity mediated by nutrient-sensing signaling pathways.

4. Implications of mitochondrial dynamics in human pathology

Defects in mitochondrial dynamics lead to mitochondrial dysfunction, which is an unequivocal culprit in the pathogenesis of various human diseases (Table 2). Some disorders linked to mitochondrial dynamics are caused by monogenic mutations that directly alter fusion or division activity, while many other diseases associated with mitochondrial dynamics result from diverse environmental and genetic factors, such as cardiovascular diseases, neurodegenerative diseases, and metabolic disorders.

4.1. Mutations in mitochondrial dynamics genes as the primary cause of neurodegenerative diseases

The importance of mitochondrial dynamics in the neural system was first corroborated by genetic studies that identified mutations of mitochondrial fission and fusion machinery in human neurodegenerative diseases (Alexander et al., 2000; Delettre et al., 2000; Kijima et al.,

2005; Waterham et al., 2007; Zuchner et al., 2004). A mutation in *MFN2* causes Charcot-Marie-Tooth Type 2A (CMT2A) disease with clinical manifestations of the peripheral neuropathy (Vance, 2000; Zuchner et al., 2004). According to the functions of *MFN2*, several mechanisms have been proposed to be responsible for the pathogenesis of CMT2A. Firstly, due to the importance of mitochondrial fusion in maintaining mtDNA integrity, inhibiting this process results in mtDNA depletion and thus dysfunction of mitochondrial respiratory chain, which renders neurons more susceptible to death (Chen et al., 2005; Ono et al., 2001). In line with this hypothesis, the absolute mtDNA copy numbers in CMT2A patient skeletal muscle biopsies were reduced approximately two-fold when compared to healthy controls (Vielhaber et al., 2013). *MFN2* is also related to the pathogenesis of CMT2A through compromised mitochondrial transport in nerve cells. This is currently considered the major cause of neuronal degeneration in CMT2A, according to findings from previous animal studies (Baloh et al., 2007; El Fissi et al., 2018; Misko et al., 2010). Mutation of *MFN2* disrupts proper mitochondrial positioning which undermines the ability of mitochondria to fulfill the local energetic needs within axons, thus resulting in axonal degeneration (Misko et al., 2010).

Heterozygous mutation in the fusion gene *OPA1* results in autosomal dominant optic atrophy (DOA), an inherited neuropathy characterized by preferential loss of retinal ganglion cells (RGCs) and optic nerve fibers (Delettre et al., 2000; Votruba et al., 1998). In addition, heterozygous *OPA1* mutations are also associated with other symptoms including deafness, ataxia, axonal sensory-motor polyneuropathy, and mitochondrial myopathy (Amati-Bonneau et al., 2008; Hudson et al., 2008; Payne et al., 2004). Although different individuals with autosomal dominant optic atrophy may present varied clinical phenotypes, visual loss resulting from the retinal ganglion cells degeneration is the major clinical manifestation (Yu-Wai-Man and Newman, 2017).

In contrast to neuropathies resulting from mutations in *MFN2* and *OPA1*, which are late-onset disorders and have localized pathogenic effects, patients with dominant-negative mutation in *DRP1* present more severe and fatal clinical abnormalities with a prenatal-onset including microcephaly, abnormal brain development, optic atrophy, and hypoplasia (Waterham et al., 2007). This result suggests that mitochondrial fission plays a more critical role than does mitochondrial fusion in human health, which may be due to the fact that *DRP1* mutations also led to impaired peroxisome fission which additionally exacerbates the severity of symptoms (Waterham et al., 2007).

4.2. Neurodegenerative diseases associated with imbalanced mitochondrial dynamics

Abnormal, fragmented mitochondrial networks have been implicated in many other neurodegenerative diseases that are not primarily caused by mutations in fission- or fusion-related genes, such as Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD). For example, loss of *PINK1* or *Parkin* in human SH-SY5Y cells, associated with PD, results in excessive mitochondrial fragmentation mediated by *DRP1* (Dagda et al., 2009; Lutz et al., 2009). Blocking the fission process attenuates dopaminergic neuronal degeneration and restored striatal dopamine release in both a *PINK1* deletion PD mouse model and an environmental PD mouse model (Rappold et al., 2014). In addition, beta-amyloid, believed to be the primary cause of Alzheimer's disease, increases neuronal nitric oxide, which triggers mitochondrial fragmentation and neuronal damage by activating *DRP1* through S-nitrosylation (Cho et al., 2009). Consistent with this observation, the level of S-nitrosylated *DRP1* is also elevated in brains of human AD, suggesting mitochondrial fragmentation as a contributor to the pathogenesis of AD (Cho et al., 2009). Accordingly, inhibiting mitochondrial fission pharmacologically alleviates beta-amyloid-caused neuronal apoptosis (Kim et al., 2016). In addition, Tau and mutant huntingtin were shown to directly interact with and activate *DRP1*, consequently augmenting fission and sensitizing neurons to

apoptosis observed in patients with HD (Shirendeb et al., 2011) or AD (Manczak and Reddy, 2012). Collectively, these data highlight mitochondrial fragmentation as an important contributor to the pathogenesis of these neurodegenerative diseases and suggest that restoring homeostasis of mitochondrial morphology genetically and/or pharmaceutically is a potentially viable and appealing therapeutic strategy to treat these diseases.

4.3. Mitochondrial dynamics and cardiovascular disease

The heart is rich in mitochondria due to high energy demands for the excitation-contraction cycle. Decreases in mitochondrial density, OXPHOS, and mitochondrial fusion proteins including MFN1 and OPA1 have been found in failing hearts in humans (Ahuja et al., 2013; Rosca and Hoppel, 2010). Conditional combined *Mfn1/Mfn2* knockout in adult mouse heart leads to overall fragmentation of the mitochondrial network, dysfunction of cardiomyocytes, and eventually overt heart failure after seven to eight weeks of tamoxifen induction of the knockout (Jin et al., 2011). Interestingly, although MFN1 and MFN2 share high sequence similarities in mammals and are both abundant in the heart (Santel and Fuller, 2001), individual knockout of either *Mfn1* or *Mfn2* leads to distinct effects on cardiac functions (Papanicolaou et al., 2011, 2012b). Conditional knockout of *Mfn1* in mouse heart results in fragmented mitochondria. However, in this context, mitochondrial respiration is normal and the hearts of *Mfn1* knockout mice present no gross abnormalities (Papanicolaou et al., 2012b). In contrast, *Mfn2*-deficient mice develop modest cardiac hypertrophy and *Mfn2* deficiency in adult cardiomyocytes leads to mitochondrial enlargement with increased production of mitochondrial ROS (Papanicolaou et al., 2011). These results indicate that, in addition to mitochondrial fragmentation in the heart, some functions of MFN2 possibly contribute to heart dysfunction, such as its role in ER-mitochondria tethering and mitophagy, as discussed above. In addition, heterozygous *Opa1* mice develop late-onset cardiomyopathy (Chen et al., 2012). These results point to the importance of mitochondrial fusion genes in preserving heart function.

In addition to heart dysfunction resulting from fusion deficiency, mitochondrial fission-related genes are also essential to the heart. Conditionally ablating *Drp1* in adult mouse hearts results in lethal dilated cardiomyopathy and cardiomyocyte necrosis by generating enlarged mitochondria and activating mitochondrial permeability transition pores (Song et al., 2015). These data illustrate the significance of balanced fission and fusion in heart functions and the loss of either one will result in heart dysfunction.

Growing evidence supports the importance of balanced mitochondrial dynamics in cardiac functions. A concomitant depletion of *Mfn1* and a mitochondrial fission gene (*Mff*), remarkably rescued *Mff* deletion-caused mitochondrial respiration deficiency, cardiac-associated dysfunction, and lifespan shortening in mice (Chen et al., 2015). In addition, mice with *Mfn1/Mfn2/Drp1* triple knockout specifically in the heart have less severe cardiac defects and survive longer compared to mice with respective cardiac knockout of *Mfn1/Mfn2* and *Drp1* (Song et al., 2017). In summary, these data demonstrate that fusion and division coordinately sustain cardiac function and suggest that preserving the balance of fusion and division might be a promising therapeutic strategy for cardiac disease.

4.4. Mitochondrial dynamics and metabolic disorders

Mitochondrial morphology reacts to distinct metabolic cues. A growing body of evidence suggests that mitochondrial network dysfunction is tightly associated with metabolic disorders including obesity, insulin resistance, and diabetes (Fig. 3). For example, in obese Zucker rats and obese humans, skeletal muscle mitochondria are smaller and fragmented, along with reduced *Mfn2* expression (Bach et al., 2003). *Mfn2* deletion in mouse liver evokes glucose intolerance

and results in insulin resistance in both liver and muscle (Sebastian et al., 2012). Studies in rat models show that *Mfn2* overexpression improves insulin sensitivity by reducing accumulation of lipid intermediates in skeletal muscle (Zhang et al., 2013) and ameliorates high-fat diet induced insulin resistance in liver (Gan et al., 2013). In line with this observation, an increase in fission protein DRP1 and mitochondrial fragmentation has been also reported in skeletal muscle from mice with diet- or leptin-deficient-induced obesity (Jheng et al., 2012). Inhibiting fission by treating diabetic mice with the DRP1 inhibitor mdivi-1 partly alleviates glucose-induced mitochondrial malfunction (Huang et al., 2015). Although these findings suggest the association between mitochondrial fragmentation and development of insulin resistance, the mechanism underlying the alteration of mitochondrial morphology during the development of insulin resistance or metabolic syndromes is still elusive. In this context, more studies should focus on the regulation of mitochondrial dynamics proteins at the transcriptional, translational, and posttranslational levels in an attempt to answer if they are regulated by pathways of insulin signaling, and glucose and fatty acid metabolism.

In contrast with metabolic inflexibility induced by *Mfn2* deletion in mouse liver and skeletal muscle, adipose-specific *Mfn2* knockout mice were resistant to high-fat diet-induced insulin resistance (Boutant et al., 2017). These results suggest that the functions of MFN2 in energy homeostasis and metabolism are highly context dependent. Similarly, deletion of *Mfn1* in mouse liver protects against insulin resistance induced by high-fat diet, despite mitochondrial fragmentation, which is opposite to the findings obtained in mice with *Mfn2* deletion in the liver (Kulkarni et al., 2016). This discrepancy can be partly reconciled by considering that in addition to mediating fusion, MFN2 is also involved in tethering the ER and mitochondria (de Brito and Scorrano, 2009). Indeed, liver-specific ablation of *Mfn2* rather than *Mfn1* in mice evokes ER stress (Kulkarni et al., 2016; Sebastian et al., 2012). Future studies are warranted to elucidate the precise role of mitochondrial dynamics proteins in different tissues in relation to these metabolic disorders.

5. Concluding remarks

Mitochondria are major crossroads of catabolic and anabolic metabolism and signaling hubs that integrate diverse cellular signals to regulate cell growth, differentiation, vitality, and death. As such, mitochondrial dysfunction is one of the classical hallmarks of aging (Lopez-Otin et al., 2013). Although knowledge of how mitochondria affect and are affected by aging has grown considerably in recent decades, there is still much to untangle with regards to how the mitochondria influences health and aging in complex organisms. Considering this question from the perspective of mitochondria network dynamics, the following conclusions emerge: (1) mitochondrial form and function are closely interlinked, and the dynamic nature of mitochondrial morphology permits mitochondria to adjust their functions in response to intrinsic and extrinsic signals through a prompt morphological rearrangement; (2) divergent cellular signaling pathways that control the rate of aging such as insulin/IIS, LET-363/TOR, AMPK, and mitochondrial dysfunction-mediated longevity pathways correspond to specific mitochondrial forms, ranging from hyperfused to fragmented patterns (Chaudhari and Kipreos, 2017; Lee et al., 2003; Morita et al., 2017; Trewin et al., 2018); (3) mitochondrial fission and fusion are integral to mitochondrial behavior, but the significance of each process to mitochondrial and cellular functions is highly context-dependent. We propose that remodeling mitochondrial morphology is likely the common strategy adopted by various signaling pathways to impinge on mitochondrial activities and therefore exert their influences on cellular bioenergetic homeostasis. However, more questions remain regarding the regulation of mitochondrial dynamics by cellular signaling pathways and even more so when considering implications in health and aging (see Outstanding Questions). In this regard, future investigations should focus on revealing regulatory mechanisms

underpinning mitochondrial morphological changes in aging and aging-related pathologies as well as the downstream implications of the mitochondrial network in aging contexts. A better understanding of these principles will provide a promising opportunity to slow the progress of age-related diseases by targeting the mitochondrial network.

Outstanding questions:

- 1 A large body of evidence connects mitochondrial dynamics to aging in yeast. However, some findings, such as lifespan shortening when fission and fusion are both impaired, are opposite to those observed in multicellular organisms. How can we reconcile this for processes we expect to be conserved?
- 2 Besides enzymes such as AMPK, PKA, and calcineurins that alter the mitochondrial network by directly modifying mitochondrial dynamics proteins in mammalian cells, are there more enzymes, yet unknown, that also participate in this process?
- 3 How do mitochondrial morphology and dynamics proteins change with age in mammals? Are these changes cell-type or tissue-specific?
- 4 Are changes throughout lifespan aging-associated defects or protective, adaptive, mechanisms? Accordingly, what are the upstream signaling pathways that regulate these mitochondrial network alterations during aging?
- 5 What are the downstream metabolic effects of mitochondrial network remodeling in different longevity paradigms such as insulin/IIS, LET-363/TOR, AMPK, and mitochondria dysfunction-mediated longevity pathways?
- 6 What role does inter-organelle communication play during aging, such as that between mitochondria, ER, and lysosomes? How are mitochondrial dynamics related to these processes?

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Article 2:
**DRP1 haploinsufficiency
attenuates cardiac
ischemia/reperfusion injuries**

DRP1 haploinsufficiency attenuates cardiac ischemia/reperfusion injuries

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Abstract:	<p>Mitochondrial dynamics is a possible modulator of myocardial ischemia/reperfusion injuries (IRI). We previously reported that mice partially deficient in the fusion protein OPA1 exhibited higher IRI. Therefore, we investigated whether deficiency in the fission protein DRP1 encoded by Dnm1l gene would affect IRI in Dnm1l +/- mouse. After baseline characterization of the Dnm1l +/- mice heart, using echocardiography, electron microscopy, and oxygraphy, 3-month-old Dnm1l +/- and wild type (WT) mice were exposed to myocardial ischemia/reperfusion (I/R). The ischemic area-at-risk (AAR) and area of necrosis (AN) were delimited, and the infarct size was expressed by AN/AAR. Proteins involved in mitochondrial dynamics and autophagy were analyzed before and after I/R. Mitochondrial permeability transition pore (mPTP) opening sensitivity was assessed after I/R. Heart weight and left ventricular function were not significantly different in 3-, 6- and 12-month-old Dnm1l +/- mice than in WT. The cardiac DRP1 protein expression levels were 60% lower, whereas mitochondrial area and lipid degradation were significantly higher in Dnm1l +/- mice than in WT, though mitochondrial respiratory parameters and mPTP opening did not significantly differ. Following I/R, the infarct size was significantly smaller in Dnm1l +/- mice than in WT (34.6±3.1% vs. 44.5±3.3%, respectively; p<0.05) and the autophagic markers, LC3 II and P62 were significantly increased compared to baseline condition in Dnm1l +/- mice only. Altogether, data indicates that increasing fusion by means of Dnm1l deficiency was associated with protection against IRI, without alteration in cardiac or mitochondrial functions at basal conditions. This cardioprotection mechanism may be related to an increased mitophagy process.</p>
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Dear Editor in Chief,

I am writing to you to request your consideration of our manuscript entitled “DRP1 haploinsufficiency attenuates cardiac ischemia reperfusion injuries” for publication in PLOS ONE.

Due to their major cell functions, mitochondria are critical structures involved in ischemia reperfusion injuries (IRI). Mitochondria are dynamic structures changing morphology by fission and fusion under the action of proteins anchored in the inner or outer mitochondrial membranes. Myocardial IRI are associated with an imbalance in mitochondrial dynamics in favor of fission. In previous *in vivo* experiments, our group observed a larger infarct size in mice with partial deficiency of the fusion protein Opa1 exposed to myocardial I/R. With the opposite approach, targeting the fission protein Drp1, we observed that increasing mitochondrial fusion by means of DRP1 deficiency was associated with protection against IRI. This cardioprotection mechanism appeared to be related to an increased mitophagy process. Therefore, modulating mitochondrial dynamics is a promising topic of research to limit IRI.

We would like to state that the manuscript, or part of it, has neither been published nor is currently under consideration for publication by any other journal. All authors have read and approved the manuscript, and they have no conflicts of interest to disclose.

Yours sincerely,

D Prunier, MD, PhD

1 **DRP1 haploinsufficiency attenuates cardiac ischemia/reperfusion injuries**

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23 **Abbreviated title:** DRP1 and myocardial I/R injuries

24

25

26

1 **ABSTRACT**

2 Mitochondrial dynamics is a possible modulator of myocardial ischemia/reperfusion injuries
3 (IRI). We previously reported that mice partially deficient in the fusion protein OPA1
4 exhibited higher IRI. Therefore, we investigated whether deficiency in the fission protein
5 DRP1 encoded by *Dnm1l* gene would affect IRI in *Dnm1l*^{+/-} mouse. After baseline
6 characterization of the *Dnm1l*^{+/-} mice heart, using echocardiography, electron microscopy,
7 and oxygraphy, 3-month-old *Dnm1l*^{+/-} and wild type (WT) mice were exposed to myocardial
8 ischemia/reperfusion (I/R). The ischemic area-at-risk (AAR) and area of necrosis (AN) were
9 delimited, and the infarct size was expressed by AN/AAR. Proteins involved in mitochondrial
10 dynamics and autophagy were analyzed before and after I/R. Mitochondrial permeability
11 transition pore (mPTP) opening sensitivity was assessed after I/R. Heart weight and left
12 ventricular function were not significantly different in 3-, 6- and 12-month-old *Dnm1l*^{+/-} mice
13 than in WT. The cardiac DRP1 protein expression levels were 60% lower, whereas
14 mitochondrial area and lipid degradation were significantly higher in *Dnm1l*^{+/-} mice than in
15 WT, though mitochondrial respiratory parameters and mPTP opening did not significantly
16 differ. Following I/R, the infarct size was significantly smaller in *Dnm1l*^{+/-} mice than in WT
17 (34.6±3.1% vs. 44.5±3.3%, respectively; p<0.05) and the autophagic markers, LC3 II and
18 P62 were significantly increased compared to baseline condition in *Dnm1l*^{+/-} mice only.
19 Altogether, data indicates that increasing fusion by means of *Dnm1l* deficiency was
20 associated with protection against IRI, without alteration in cardiac or mitochondrial functions
21 at basal conditions. This cardioprotection mechanism may be related to an increased
22 mitophagy process.

23 **Keywords:** ischemia-reperfusion injuries, mitochondrial dynamics, mitochondrial fission,
24 DRP1

25

26

27

1 INTRODUCTION

2 Ischemic heart disease, and more specifically myocardial infarction (MI), is still the leading
3 cause of morbidity and mortality in developed countries (12). Timely reperfusion during
4 myocardial infarction is crucial for the salvage of the ischemic myocardium, but paradoxically
5 leads to ischemia-reperfusion injuries (IRI) that account for the final myocardial damage(23).
6 Due to their major cell functions, such as ATP synthesis, calcium homeostasis, and cell
7 death/survival mechanisms, mitochondria are critical structures involved in IRI (9, 13).
8 Mitochondria are dynamic structures changing morphology by fission and fusion
9 mechanisms under the action of proteins anchored in the inner or outer mitochondrial
10 membranes (24). This mitochondrial dynamics proves essential to maintaining the
11 mitochondrial network integrity and appears of great relevance in IRI (11, 13). Mitochondrial
12 fusion orchestrated by optic atrophy 1 protein (OPA1) and mitofusin 1 and 2 (MFN1, 2) leads
13 to elongated mitochondria, enabling exchanges of mitochondrial matrix proteins and
14 mitochondrial DNA. In contrast, mitochondrial fission results in smaller and fragmented
15 mitochondria and is orchestrated by the GTPase dynamin related protein-1 (DRP1). DRP1 is
16 a cytosolic protein that translocates to the mitochondrial outer membrane following post-
17 translational modifications. By means of oligomerization and constriction, DRP1 leads to
18 mitochondrial fission through its interaction with outer mitochondrial membrane receptors,
19 such as the human fission protein factor 1 (hFIS1), mitochondrial fission factor (Mff), and
20 mitochondrial dynamics proteins of 49 and 51KDa (MiD49 and MiD51). Fission is an
21 essential step in the mitophagy mechanism, enabling the segregation of unrecoverable
22 mitochondria to initiate their elimination (8).

23 Myocardial IRI are associated with an imbalance in mitochondrial dynamics in favor
24 of fission (2, 14). Thus, modulating mitochondrial dynamics is a topic of intense research
25 designed to limit IRI. In previous *in vivo* experiments, we observed a larger infarct size in
26 mice with partial deficiency of the fusion protein Opa1 exposed to myocardial I/R than in that
27 of wild-type (11). This finding was consistent with an *in vitro* study in which simulated
28 ischemia of the cardiomyoblast cell line H9C2 cells with Opa1 *shRNA* increased

1 mitochondrial fragmentation and cell death (5). Moreover, a mild Opa1 overexpression was
2 found to protect the mice hearts from ischemic damage (19). The opposite approach,
3 consisting of inhibiting fission by pharmacologically targeting DRP1's activity or *Dnm1l*'s
4 expression (10, 18), provided controversial results. A cardioprotective effect has been
5 observed in a few studies and attributed to the inhibition of mitochondrial permeability
6 transition pore (mPTP) opening (7, 14). Other studies reported increased IRI, which were
7 attributed to mitochondrial autophagy inhibition (10).

8 Therefore, this study sought to investigate whether DRP1 deficiency influences
9 myocardial IRI *in vivo* in a deficient *Dnm1l* mice model.

10

11 **METHODS**

12 *Mouse model*

13 DRP1 is encoded by the Dynamin 1 like gene (*Dnm1l*). Previously described
14 heterozygous *Dnm1l* knockout (*Dnm1l*^{+/-}) mice (22) were obtained from Professor Hiromi
15 Sesaki at the Johns Hopkins University. Mice were kept using standard light cycles, with
16 food and water available ad libitum. All animal work was performed according to the
17 European Community Guiding Principles in the care and use of animals (Directive
18 2010/63/UE; Décret n°2013-118). Authorizations to conduct animal experiments were
19 obtained from the MENESR, *Ministère de l'Education Nationale de l'Enseignement*
20 *Supérieur et de la Recherche* (APAFIS#4514-2016031415098201 v2).

21

22 *Echocardiography*

23 Transthoracic echocardiography (TTE) was performed using an ultrasound system
24 (Vivid 7 Pro, General Electric Medical Systems) on anesthetized mice (ketamine 60-
25 70mg/Kg, intraperitoneally), as previously described (15). Left ventricular end diastolic

1 diameter (LVEDD), left ventricular end systolic diameter (LVESD), and fractional shortening
2 (FS) were determined using two-dimensional (2D) M-mode echocardiography.

3 *Mitochondrial morphology by transmission electron microscopy*

4 Samples of left ventricular (LV) papillary muscle were prepared for EM, as previously
5 described (22). Briefly, the hearts were fixed using a retrograde perfusion with 2%
6 glutaraldehyde in cacodylate buffer (100mM sodium cacodylate and 2mM MgCl₂; pH 7.3).
7 Ultra-thin longitudinal sections of 60nm were cut and examined using an electron
8 microscope, with 20,000x-magnified images by means of the software Image J, designed to
9 assess mitochondrial number and area. In total, 60 fields on average were analyzed per
10 group for mitochondrial morphology.

11

12 *Respirometric investigation of mitochondrial function*

13 Preparation of permeabilized cardiac fibers, previously described (16), was
14 conducted in order to study mitochondrial function *in situ*. The respiratory rates were
15 recorded at 30°C in 2mL glass chambers using a high-resolution Oxygraph respirometer
16 equipped with a Clark oxygen sensor (Oroboros, Innsbruck, Austria) and analyzed by means
17 of DATLAB Analysis software (OROBOROS, Austria).

18 Respiration was initiated with complex I-dependent substrates (5mM malate/ 2.5mM
19 pyruvate). Complex I-coupled state 3 respiration was measured by adding saturating ADP
20 (1mM), followed by 10mM succinate, enabling the full TCA cycle operation to occur, with
21 maximal coupled respiration sustained by both complexes I+II. Next, 2.5µM rotenone was
22 injected in order to obtain the complex II-coupled state 3 respiration. Oligomycin (8µg/mL)
23 was then added to determine the uncoupled state 4 respiration. Finally, FCCP (1 µM) was
24 added to further control the fiber permeabilization.

1 The second experiment was initiated with malate and palmitoylCoA/carnitine as
2 substrates (2.5mM malate/ 40μM PalmitoylCoA/ 1mM carnitine), following which 1mM ADP
3 was added in order to investigate the beta-oxidation under phosphorylating condition. Next,
4 antimycin (2μg/mL) was added to inhibit complex III. The experiment was continued to
5 assess complex IV-coupled state 3 respiration, following which complex IV inhibitors (1 mM
6 KCN/ 2 mM azide) were added.

7 The last experiment was conducted in order to evaluate the coupling of oxidative
8 phosphorylation with mitochondrial creatine kinase. Fibers were exposed to increasing ADP
9 concentrations, either in the presence or absence of creatine (20mM), in order to stimulate
10 the creatine kinase system; the ADP-stimulated respiration was plotted above basal oxygen
11 consumption (V_0) in order to determine the maximal respiration rate (V_{max}).

12

13 *In vivo ischemia-reperfusion experiments and infarct size assessment*

14 Overall, 3-month-old male *Dnm1^{f/f}* mice and their littermates were submitted to
15 myocardial I/R *in vivo*. *Dpr1^{f/f}* and WT mice were anesthetized by means of intraperitoneal
16 sodium pentobarbital injections (80mg/Kg; Ceva Santé Animale) and received an injection of
17 heparin (100IU/Kg, Heparine Choay[®], Sanofi aventis, Paris, France) prior to thoracotomy.
18 Myocardial I/R was achieved by temporarily occluding the left coronary artery, then releasing
19 the occlusion as previously described (11) (12). The duration of ischemia was 30 minutes,
20 and that of reperfusion 24 hours.

21 To delimit the ischemic area-at-risk (AAR) and area of necrosis (AN), Evans Blue 4%
22 (Sigma-Aldrich Co[®], Missouri, USA) was injected through the left-ventricular apex.
23 Thereafter, the heart sections were incubated with 2,3,5-triphenyltetrazolium chloride (TTC)
24 (Sigma-Aldrich Co[®], Missouri, USA) and fixed in 4% paraformaldehyde for 24 hours, as
25 previously described (11). The AN (white), AAR (not blue), and total left-ventricular (LV)

1 areas from both sides of each section were measured using the software ImageJ 1.47.
2 AAR/LV and AN/AAR were expressed as percentages, as previously reported (11).

3

4 *Western Blot Analysis*

5 Three-month-old male and female *Dnm1^{+/+}* mice and their littermates were employed.
6 The methods applied for immunoblot preparation were previously described (11). Protein
7 expression was assessed at basal condition and after the *in vivo* I/R procedure. Overall, 30-
8 50µg of total proteins was separated by SDS-PAGE and transferred to a nitrocellulose or
9 PVDF membrane. The membranes were incubated overnight at 4°C with primary antibodies,
10 and thereafter, with appropriate secondary antibodies conjugated to horseradish peroxidase
11 (Santa Cruz Biotechnology®, Texas, USA). The antibodies used consisted of OPA1, DRP1
12 (BD Transduction Laboratories®; 1/1000), MFN2 (Sigma-Aldrich®; 1/2000), FIS1 (Santa Cruz
13 Biotechnology®, 1/500), LC3B (Enzo Life Sciences; 1/1000), p62 (Enzo Life Sciences;
14 1/1000), and GAPDH (Sigma-Aldrich®; 1/20000) was employed as a loading control.

15

16 *Metabolomic Analysis*

17 We applied a targeted, quantitative metabolomic approach to heart tissue extracts by
18 using the Biocrates AbsoluteIDQ p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria),
19 as previously described (4). The ratio of short-chain acylcarnitines to free carnitine
20 (C2+C3/C0) as a measure of overall beta-oxidation activity, and the ratio of long-chain
21 acylcarnitines to free carnitine (C16+C18/C0) as activity of carnitine palmitoyltransferase 1
22 (CPT1) were quantified.

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1 *Calcium Retention Capacity (CRC)*

2 The mitochondrial permeability transition pore (mPTP) opening was assessed by
3 mean CRC, as previously described (1). In brief, extra-mitochondrial Ca²⁺ concentration was
4 estimated using Calcium GreenTM-5N (Life Technologies). Measurements were carried out
5 on isolated cardiac mitochondria from *Dnm1*^{+/-} after I/R were performed with or without the
6 adjunction of cyclosporine A (1μM). CRC was expressed as nmoles Ca²⁺/mg of proteins.

7 *Statistical analysis*

8 Data have been expressed as mean±standard error of the mean (SEM). The statistical
9 significance of the difference between the *Dnm1*^{+/-} and WT mice groups was estimated
10 using the Student's *t*-test or non-parametric Mann-Whitney test, as appropriate, with *p*-
11 values <0.05 considered statistically significant.

12

13 **RESULTS**

14 *Anatomical characteristics and heart function of Dnm1*^{+/-} *mice*

15 We characterized the cardiac weight, LV size, and LV function in 3- 6- and 12-month-
16 old *Dnm1*^{+/-} and WT mice. The heart rate recorded at the time of echocardiography was not
17 significantly different between groups of the same age (591±30 bpm in WT vs. 574±15 bpm
18 in *Dnm1*^{+/-} at 3 months; 570±15 bpm in WT vs. 585±16 bpm in *Dnm1*^{+/-} at 6 months;
19 579±13 bpm in WT vs. 576±8 bpm in *Dnm1*^{+/-} at 12 months). Body, heart weights, and LV
20 size were not significantly different between WT and *Dnm1*^{+/-} mice of the same age (Figures
21 1A and 1B), nor were LVEDD and LVESD. Likewise, cardiac function represented by FS did
22 not significantly differ at any time (55±2 % in WT vs. 58±1 % in *Dnm1*^{+/-} at 3 months; 53±1
23 % in WT vs. 54±1 % in *Dnm1*^{+/-} at 6 months; 54±2 % in WT vs. 54±1 % in *Dnm1*^{+/-} at 12
24 months) (Figure 1C).

25

1 *In vivo I/R injury*

2 The 3-month-old *Dnm1^{f/f}* and WT mice were subjected to 30-min myocardial
3 ischemia followed by 24-hour reperfusion (Figure 2). The AN/AAR after I/R, evaluated using
4 Evans Blue and TTC staining, was significantly lower in *Dnm1^{f/f}* mice than in WT
5 ($34.6\pm 3.1\%$ vs. $44.5\pm 3.3\%$, respectively; $p<0.05$), whereas AAR/LV did not significantly differ
6 between the two groups.

7 *Mitochondrial dynamics protein expression*

8 Immunoblot analyses confirmed that cardiac DRP1 levels were 60% lower in 3-
9 month-old *Dnm1^{f/f}* mice than in WT (Figure 3). At baseline, cardiac levels of other proteins
10 involved in mitochondrial dynamics, such as MFN 2, OPA1, and FIS1, were unaltered in
11 *Dnm1^{f/f}* mice compared to WT.

12 After a 30-min ischemia followed by a 24-hour reperfusion, DRP1 levels tended to be
13 lower in *Dnm1^{f/f}* mice, though the difference did not achieve statistical significance (3.7 ± 0.5
14 in WT vs. 2.5 ± 0.4 in *Dnm1^{f/f}*), whereas MFN2 levels were significantly increased in *Dnm1^{f/f}*
15 mice compared to WT (2.2 ± 0.2 in WT vs. 2.8 ± 0.1 in *Dnm1^{f/f}*). Furthermore, OPA1 and FIS1
16 were not significantly different in WT and *Dnm1^{f/f}* mice (OPA1: 2.5 ± 0.3 in WT vs. 3.0 ± 0.3 in
17 *Dnm1^{f/f}*; FIS1: 1.5 ± 0.6 in WT vs. 1.4 ± 0.4 in *Dnm1^{f/f}*) (Figure 3).

18

19 *Mitochondrial morphology*

20 Electron microscopy (EM) analyses of cardiac papillary muscle were performed at 3
21 month. At baseline, the number of mitochondria in hearts per field was not significantly
22 different between *Dnm1^{f/f}* and WT mice. However the mitochondrial areas were more
23 frequently in the first quartile in WT and in the third in *Dnm1^{f/f}* mice, indicating that the
24 mitochondria were enlarged in *Dnm1^{f/f}* (Figure 4B).

1 Following I/R, EM revealed tissue sections of the papillary muscle to exhibit a disruption in
2 the mitochondrial network in *Dnm1^{+/−}* and WT mice (Figure 4A).

3 *Mitophagy*

4 In the heart, we quantified the mitophagy protein levels, microtubule-associated
5 proteins 1A/1B light chain 3B (LC3), and sequestosome-1 or ubiquitin-binding protein p62
6 from *Dnm1^{+/−}* mice and WT, under basal conditions and after I/R. Under basal conditions,
7 there was no significant difference in the protein level expression between *Dnm1^{+/−}* and WT
8 groups (Figure 5). After I/R, we observed that LC3 II protein level was increased compared
9 to basal condition in both group WT (0.09±0.01 at baseline vs. 0.43±0.05 after I/R p<0.001)
10 and *Dnm1^{+/−}* mice (0.07±0.01 at baseline vs. 0.96±0.16 after IR p<0.001). P62 protein level
11 was significantly increased after I/R in the *Dnm1^{+/−}* mice only (0.25±0.02 at baseline vs.
12 0.36±0.05 after IR p=0.038).

19

20 *Mitochondrial function*

21 *Cardiac energy metabolism of Dnm1^{+/−} mice*

22 Next, we investigated whether any change in oxidative capacity at baseline could
23 explain the protection in *Dnm1^{+/−}* mice after I/R stress. Basal and maximal phosphorylating
24 mitochondrial respirations were not significantly different between the two groups (Figure
25 6A). None of the complex I, complex II, or complex I+II (full operating TCA) substrates
26 indicated that the respiratory chain capacity was changed. However, when using
27 palmitoylCoA/carnitine as substrate, the maximal respiration was higher in mitochondria from
28 *Dnm1^{+/−}* mice than in those from WT (37.5±3.8nmol O₂/min/mg in WT vs.
29 52.3±3.0nmolO₂/min/mg in *Dnm1^{+/−}* (Figure 6C)), supporting a higher fatty acid oxidation
30 capacity in *Dnm1^{+/−}*. Therefore, we performed metabolomic analysis on *Dnm1^{+/−}* and WT
31 heart tissues. There was no significant difference between the two groups in either beta

1 oxidation activity (0.80 ± 0.04 in WT vs. 0.76 ± 0.05 in *Dnm1^{f/f}*) or CPT1 activity (0.08 ± 0.01 in
2 WT vs. 0.07 ± 0.01 in *Dnm1^{f/f}*) (Figure 6D).

3 The sensitivity of mitochondrial respiration to ADP was estimated with and without
4 creatine to study the coupling of oxidative phosphorylation with mitochondrial creatine
5 kinase. The ratio K_m for ADP without (K_{mADP}) and with creatine ($K_{mADP+creat}$) was assessed
6 with no significant difference found between the two groups (8.2 ± 2.3 in WT vs. 3.1 ± 0.6 in
7 *Dnm1^{f/f}*; $p=0.14$) (data not shown).

8 Cardiac mPTP function of *Dnm1^{f/f}* mice

9 CRC is taken as an indicator of susceptibility to mPTP opening following calcium
10 overload. We measured CRC on mitochondria isolated from the area at risk of *Dnm1^{f/f}* and
11 WT mice. Following IR, CRC was not altered in either group. CsA, a selective mPTP
12 inhibitor, tended to increase the CRC value in WT and *Dnm1^{f/f}* mitochondria after I/R,
13 though not significantly (Figure 6B).

14

15 **DISCUSSION**

16 Mitochondrial dynamics and myocardial IRI are thought to be closely related. Mitochondrial
17 fission is observed during myocardial ischemia (2–4), and mice partially deficient in the
18 fusion protein OPA1 were shown to exhibit higher IRI (11), while mild Opa1 overexpression
19 has previously been revealed to be protective (12,20). Given this context, targeting the
20 mitochondrial fission protein DRP1 appears to be an attractive therapeutic strategy to limit
21 IRI. Herein, we have reported that heterozygous deficient *Dnm1l* mice exhibited a lower IRI
22 degree when exposed to myocardial ischemia-reperfusion *in vivo*.

23 Because complete KO *Dnm1l* was shown to induce embryonic death by Day E11.5
24 (21), we investigated a heterozygous deficient *Dnm1l* model (*Dnm1^{f/f}*) (21). In these mice,
25 we observed a 60%-decreased DRP1 cardiac expression, without any change in the other
26 main proteins involved in mitochondrial dynamics. Importantly, no alteration of either cardiac

1 morphology or function was found at the ages of 3, 6, and 12 months. In a conditional
2 cardiac KO *Dnm1l* mice model, the *Dnm1l* downregulation induced at the age of 15 weeks
3 provoked a lethal dilated cardiomyopathy 8 weeks later through altered mitochondrial
4 respiration (10, 18). The mitochondrial respiratory chain capacity, evaluated with either
5 complex I, II, or with full operating TCA substrates in permeabilized cardiac fibers, was not
6 found to be altered in our *Dnm1l^{+/-}* model, although the mitochondria capacity to oxidize free
7 fatty acids, as evaluated using palmytoylCoA/carnitine as substrates, was found increased.
8 The mechanism responsible for this alteration is still unclear. Alteration in the transport of
9 long chain fatty acids through mitochondrial membrane does not appear to be involved,
10 because the CPT1 activity remained unchanged. Interestingly, opposite results were found
11 in *Opa1^{+/-}* mice, where cardiac mitochondria were revealed to be less able to oxidize lipids
12 than those of their WT littermates. Mitochondrial fission, induced by stress, is known to be
13 associated with a predominant glycolytic metabolism shift aimed to preserve the energy level
14 (20). Hence, one could suggest that the protective effect of increasing mitochondrial fusion is
15 partly related to its association with increased mitochondrial lipid oxidation occurring at the
16 time of reperfusion. More research is necessary to further understand the DRP1's role in
17 lipid oxidation regulation.

18

19 Due to its crucial role in cardiomyocyte death in the I/R setting, mPTP opening
20 sensitivity appears to be a credible target that could be modulated by mitochondrial
21 dynamics (5). To date, there are discordant data regarding this potential interaction (10, 14,
22 25). In cardiomyocytes with *Dnm1l* silencing using shRNA and in *Dnm1l*-KO mice hearts 4
23 weeks after tamoxifen injection, the mPTP opening was found to be accelerated by *Dnm1l*
24 down-regulation. Nevertheless, since all experiments were performed without I/R, their
25 extrapolation to IRI was limited (10). On the contrary, pre-treatment of HL-1 cells or adult rat
26 cardiomyocytes with the DRP1 inhibitor *Mdivi-1* delayed mPTP opening after simulated
27 I/R(14). Similarly, a recent study reported that genetic and pharmacological DRP1 inhibition

1 in adult cardiomyocytes reduced transient mPTP opening (25). In our model of partial *Dnm1l*
2 expression down-regulation, mPTP opening sensitivity was found to be unaltered, as
3 evaluated by mean *Calcium Retention Capacity* values. Therefore, the relationship between
4 cardioprotection through DRP1 deficiency and mPTP opening sensitivity was not assumed.

5
6 Previous studies have shown pharmacological DRP1 inhibition to be able to protect
7 the heart from IRI (6, 10, 14, 25). In this respect, *Mdivi-1*, an inhibitor of DRP1's activity, was
8 able to prevent IRI in cellular and animal models, when administered as a pretreatment
9 (4,5,7,21). On the contrary, a recent study revealed that partial or total cardiac
10 downregulation of *Dnm1l* exacerbated myocardial IRI (10). In a later study, the lack of *Dnm1l*
11 impaired autophagy, with accumulation of abnormal mitochondria and increased
12 cardiomyocyte deaths, was suggested to figure as a consequence. Surprisingly, other
13 authors reported that *Dnm1l* ablation in adult mice cardiomyocytes resulted in hyper-
14 mitophagy with mitochondrial-associated p62 and LC3 protein level upregulation (18).
15 Indeed, mitochondrial autophagy proves to be an essential regulator that contributes to strict
16 quality control by maintaining intracellular homeostasis in cardiomyocytes. Both fission and
17 fusion exert an impact on mitochondrial autophagy, with fusion allowing for mitochondrial
18 content exchange and functional complementation, and fission enabling mitochondria
19 enriched with damaged constituents to be isolated, thereby facilitating their degradation.
20 During this process, DRP1 facilitates the degradation of small mitochondria enriched with
21 damaged constituents (3), while MFN2 plays a crucial role in the fusion of autophagosomes
22 with lysosomes, a critical step in autophagic degradation. Hence, *Mfn2* deficiency in the
23 heart was found to result in impaired mitophagy, mitochondrial dysfunction, and cardiac
24 dysfunction (17, 26). In our work, we have observed no difference between *Dnm1l*^{+/−} and WT
25 mice under basal condition regarding LC3 and p62, two markers of mitophagy, which
26 suggests that DRP1 protein level is high enough to maintain mitochondrial autophagy. After
27 I/R, DRP1 haploinsufficiency observed under basal condition disappeared and DRP1

1 protein level was similar in *Dnm1*^{+/-} and WT littermates, suggesting that DRP1 protein level
2 increased more after I/R in *Dnm1*^{+/-} mice. Moreover, LC3 II, P62 and MFN2, three proteins
3 involved in mitophagy increased more in *Dnm1*^{+/-} mice than in their WT littermates. One
4 can suggest that the cardioprotection observed in *Dnm1*^{+/-} mice is related to two
5 consecutive mechanisms: a lower expression of DRP1 protein at the time of ischemia,
6 followed by a higher DRP increase after reperfusion, inducing more mitophagy.

7

8

9 **Limits**

10 We have used a constitutive heterozygous deficient *Dnm1* mice model designed to assess
11 *in vivo* *Dnm1*'s role in the development of IRI. As a consequence of this chronic *Dnm1*
12 deficiency, several unsuspected compensation mechanisms may have come into play, such
13 as increased mitochondrial autophagy and lipid oxidation. Moreover, the results pertaining to
14 the absence of effect on mPTP opening sensitivity must be interpreted with caution, as CsA
15 used as a positive control did not affect mPTP opening under our experimental condition.
16 Finally, cardioprotection was only investigated at 24 hours after I/R. It would be of interest to
17 assess whether *Dnm1* deficiency would similarly affect post-infarct cardiac remodeling at a
18 more advanced timepoint.

19

20 **Conclusion**

21 Increasing fusion by means of *Dnm1* deficiency is associated with protection against IRI,
22 without alteration in cardiac or mitochondrial functions at basal conditions. This
23 cardioprotection mechanism appears to be related to an increased mitophagy process.

24

25

26

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7 imaging.

8

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10 None

11

12 **Disclosure**

13 None

14

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9

10

11

1 **Figure legends**

2 **Figure 1** Anatomical characteristics and heart function of 3-, 6- and 12-month-old *Dnm1^{f/f}*
3 and their wild type littermate (WT) mice. (A) Body weight (BW). (B) Heart weight to body
4 weight (HW/BW) ratio. (C) Cardiac echocardiography, left: Left ventricle end-systolic volume
5 (LVESD), middle: LV end-diastolic volume (LVEDD), right: LV fractional shortening (FS).
6 Three months: n=6-10 WT and n=10 *Dnm1^{f/f}*; 6 months: n=7-10 WT and n=6-8 *Dnm1^{f/f}*; 12
7 months: n=6-8 WT and n=10 *Dnm1^{f/f}*.

8

9 **Figure 2** Histograms showing area necrosis (AN) as a percentage of area at risk (AAR) or
10 AAR as a percentage of the total left ventricle (LV) area (n=11-12/group) * $p < 0.05$.

11

12 **Figure 3** Baseline and post-ischemia/reperfusion (I/R) mitochondrial dynamics protein
13 expression. OPA1, MFN2, DRP1, and FIS1 expression assessed by means of western
14 blotting at baseline (n=8-10/group) and after I/R (n=4-10/group). Representative
15 immunoblots and histograms showing quantifications are represented. GAPDH was used as
16 a loading control. Values are expressed as mean \pm SEM. * $p < 0.05$.

17

18 **Figure 4** Mitochondrial morphology. (A) Representative electron microscopy (EM) images of
19 left ventricle longitudinal sections at baseline and after 30 minutes of ischemia and 24 hours
20 of reperfusion in 3-month-old *Dnm1^{f/f}* and WT mice. Scale bar: 1 μ m. (B) Mitochondrial
21 frequency represented according to the quartile distribution of the mitochondrial area.
22 Counting was performed on 60 representative EM fields per group (n=4 per group).

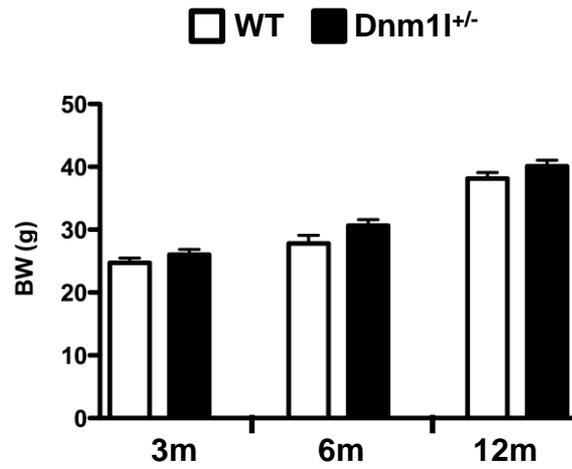
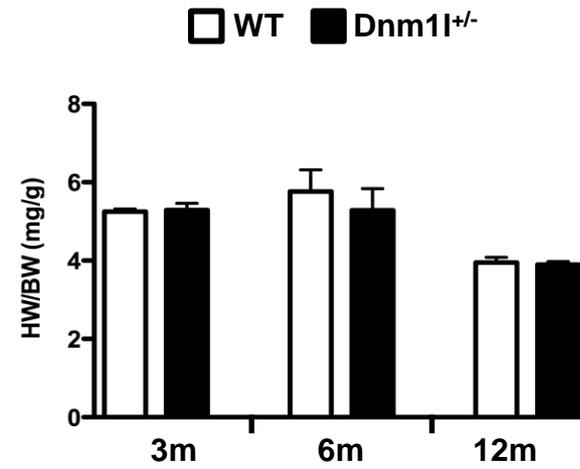
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1 **Figure 5** Representative immunoblot of autophagic response proteins. Autophagy levels
2 were assessed by quantitative analysis of autophagy markers LC3-II and p62 in *Dnm1^{f/f}* and
3 WT mice at baseline and after ischemia/reperfusion (I/R). The results are expressed as
4 ratios of protein band densities of LC3-II and p62, (n=6-10 per group). GAPDH was used as
5 a loading control. Values are mean \pm SEM.

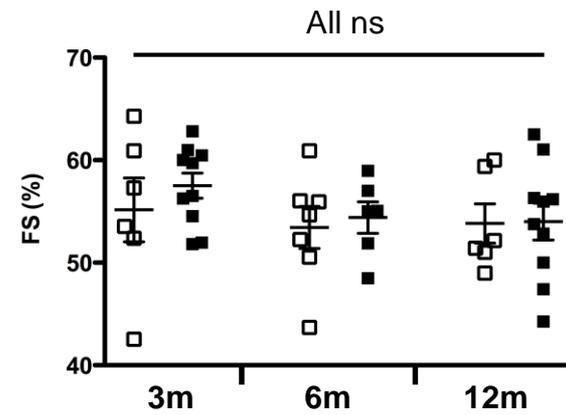
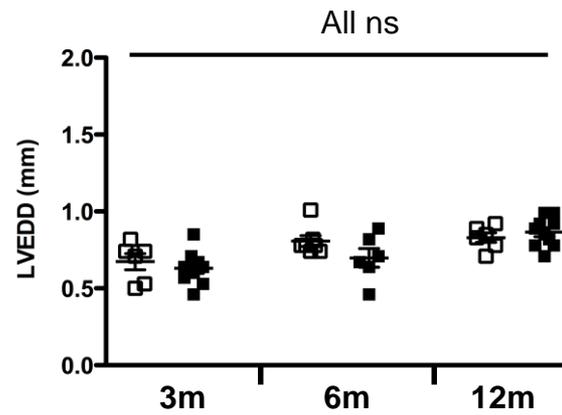
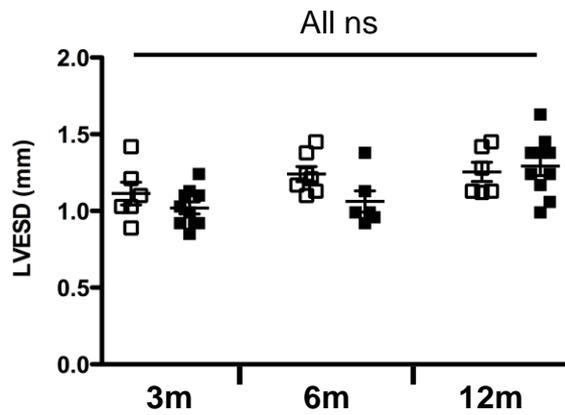
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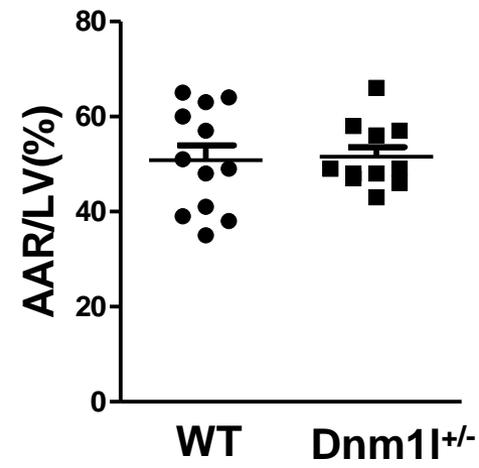
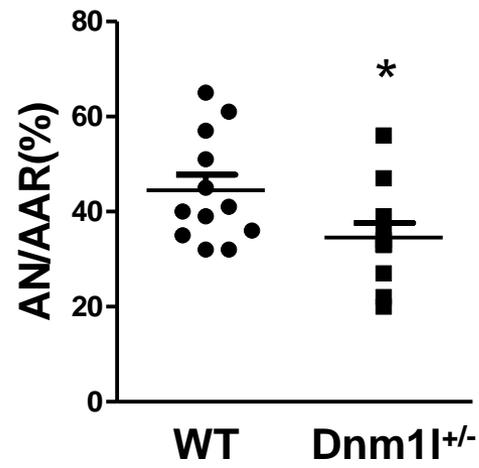
7 **Figure 6** (A) Cardiac energy metabolism and mPTP function. (A) Oxygen consumption rate
8 of permeabilized left ventricular fibers in the presence of 1mM ADP (V_{max}) with 5 mM malate
9 and 2.5mM pyruvate (complex I, CI), 10mM succinate (both CI and complex II CI + II), and
10 after complex I inhibition with 2.5 μ M rotenone (CII). Values are expressed as mean \pm SEM,
11 (n=5-6 per group). (B) CRC was measured in mitochondria isolated from the areas at risk
12 after a 45-min ischemia followed by a 15-min reperfusion. CRC measurement represented
13 the Ca^{2+} level required for mitochondrial permeability transition pore (mPTP) opening. Extra-
14 mitochondrial Ca^{2+} was detected using Calcium GreenTM-5N (Life Technologies). Results
15 were expressed as mean \pm SEM nmol Ca^{2+} mg protein⁻¹. (n=6 per group). Mitochondria
16 were isolated from 3-month-old *Dnm1^{f/f}* and WT mice. (C) Oxygen consumption rate with
17 1mM ADP and 2.5mM malate and 40 μ M palmitoylCoA/1mM carnitine. Values are expressed
18 as mean \pm SEM, n=7-8 per group; * p<0.05. (D) Metabolomic analysis of the cardiac tissue
19 in 3-month-old *Dnm1^{f/f}* and control WT mice. n=10 per group. Left: ratio of short chain
20 acylcarnitines to free carnitine (C2+C3/C0) represents a measure of overall beta-oxidation
21 activity. Right: ratio of long chain acylcarnitines to free carnitine (C16+C18/C0) represents
22 the activity of carnitine palmitoyltransferase 1 (CPT1).

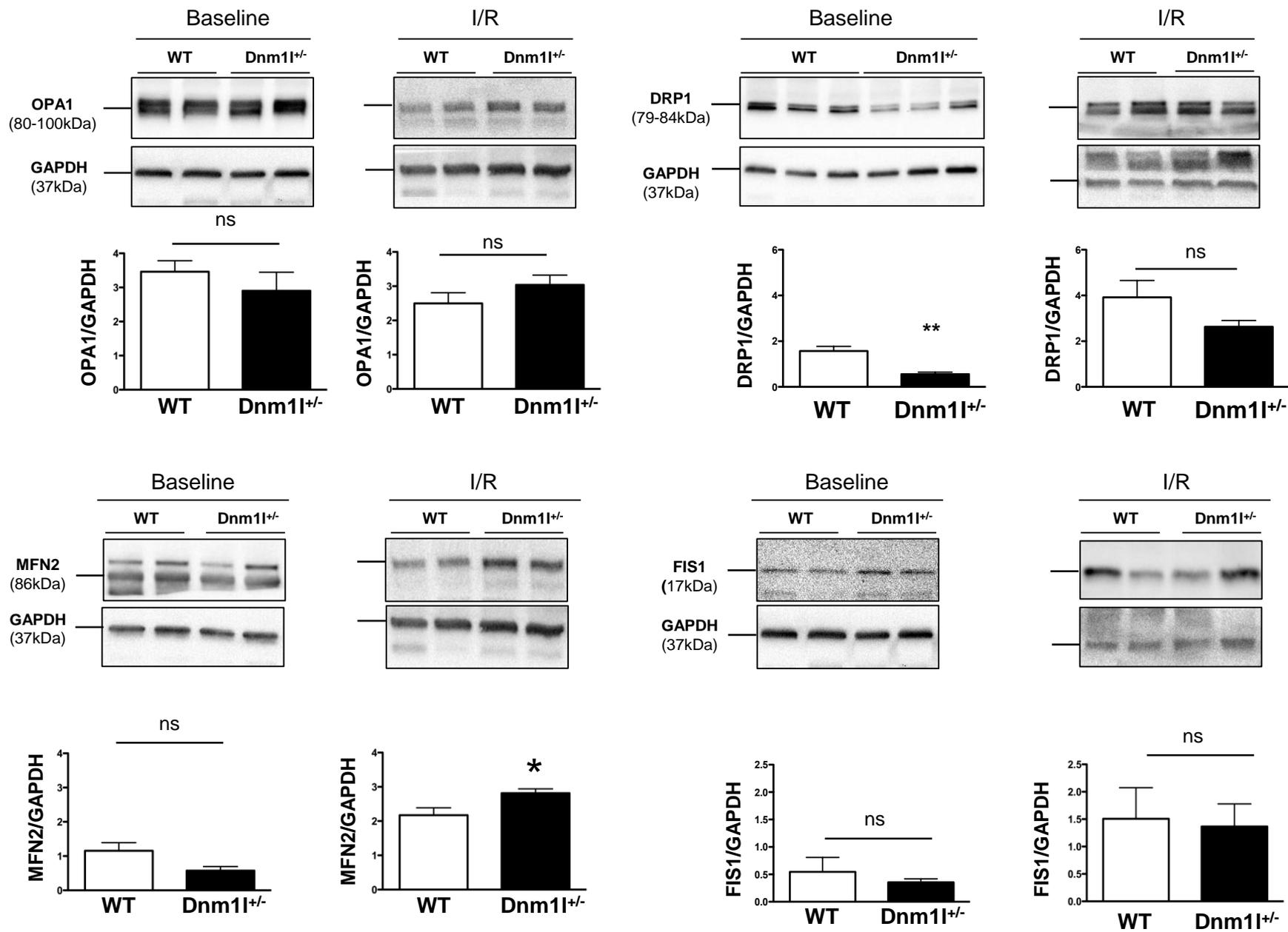
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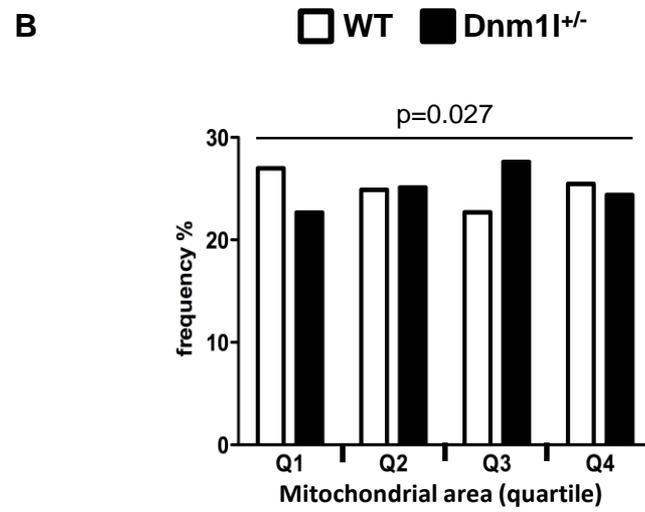
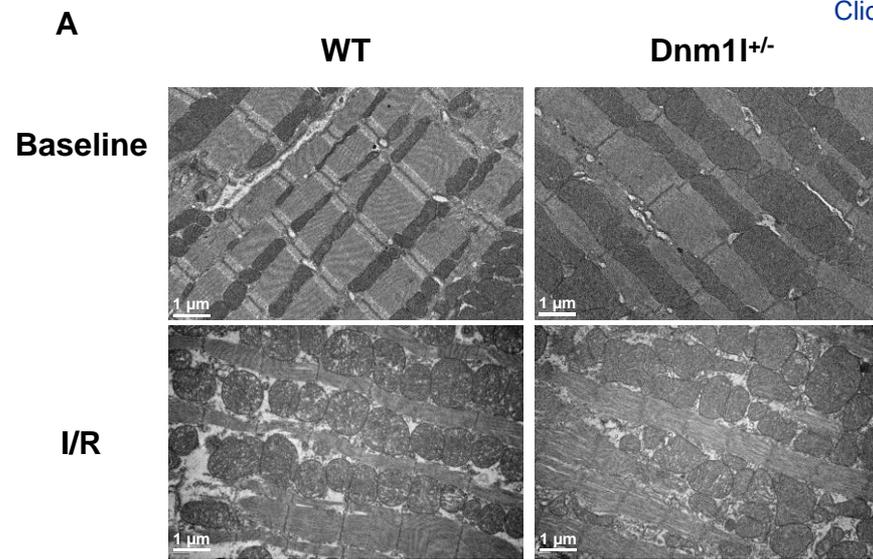
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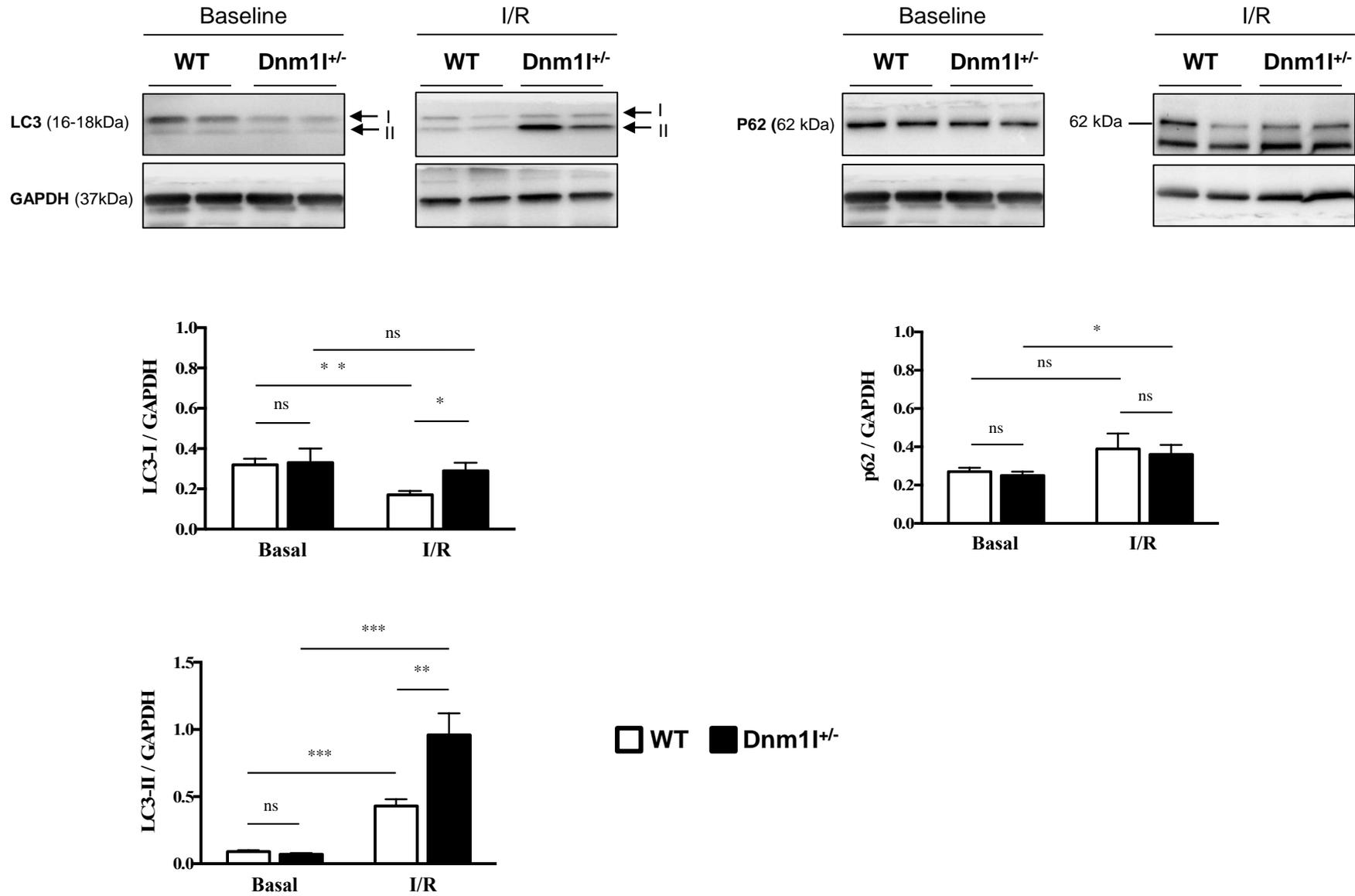
□ WT
■ Dnm1^{+/-}

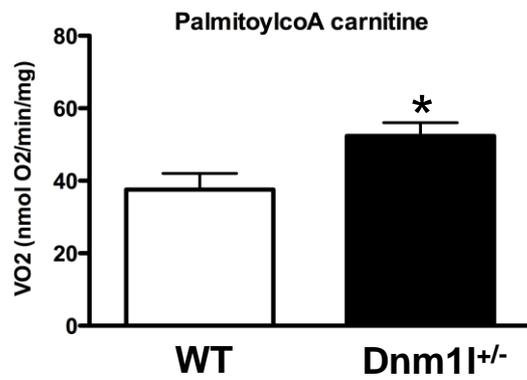
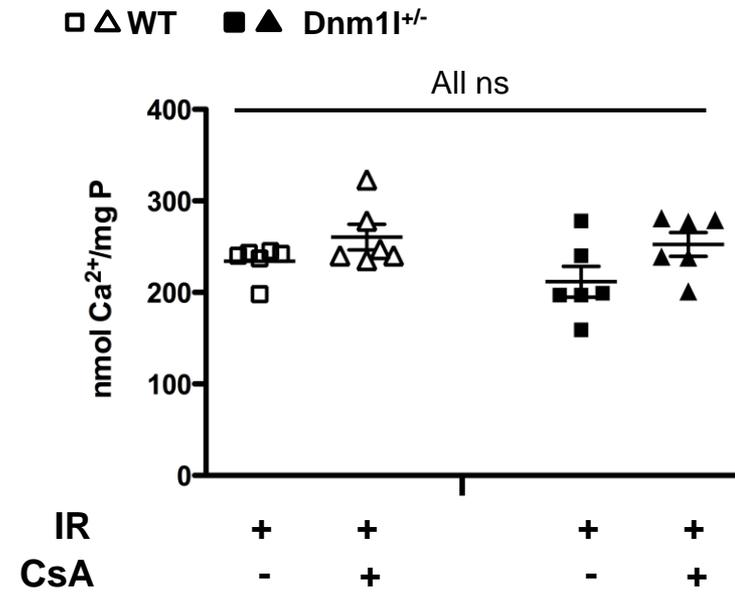
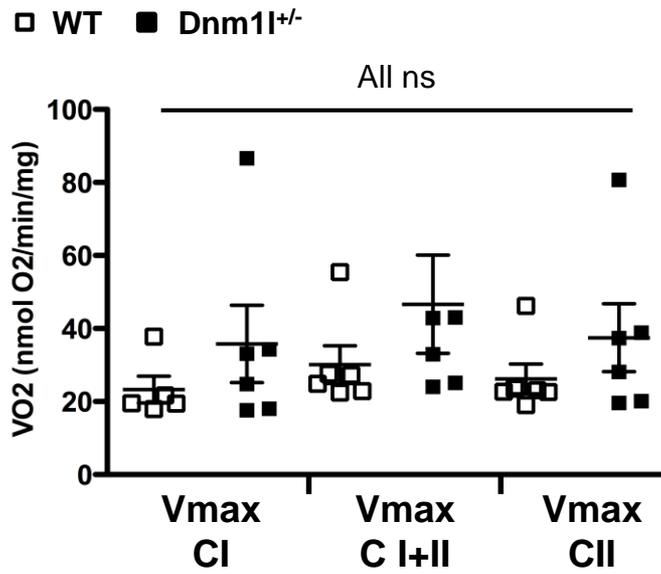
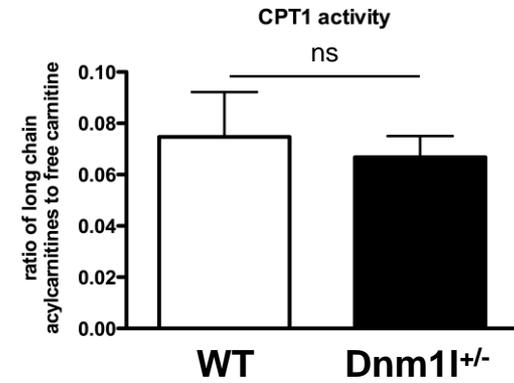
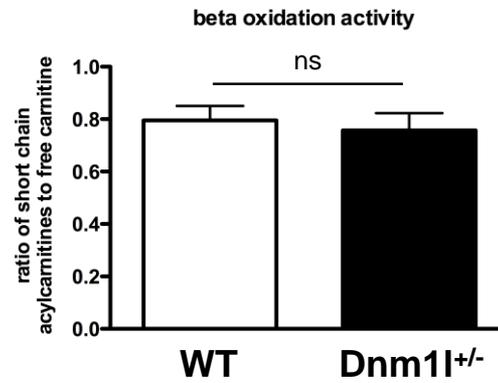










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Abstract

Abstract

Mitochondria are dynamic organelles altering their inner and outer membranes through fission and fusion mechanisms. In response to shear stress, the mitochondria in endothelial cells (ECs) continuously undergo fusion and fission mechanisms. We hypothesized that alteration in mitochondrial dynamics could alter ECs function leading to vascular damages. For that, we investigated the role of OPA1 and DRP1 deficiency in ECs and vascular homeostasis using mice lacking fusion protein OPA1 and fission protein DRP1 (heterozygous *Opa1*^{+/-} & *Drp1*^{+/-} mice). We investigated mitochondrial dynamics-related proteins expression level in ECs submitted to a disturbed shear stress (evidenced by eNOS down-regulation and endothelin-1 up-regulation) and observed a reduction in fusion protein OPA1 mRNA expression and an increase in fission protein DRP1 mRNA expression level. The opposite was observed in ECs submitted to disturbed shear stress. After acute stimulation of ECs to Histamine, cells lacking OPA1 and DRP1 protein have shown a decreased intracellular Ca²⁺ release, compared to control cells. Besides, these cells showed a decreased capability of migration compared to control cells. As shear stress determines atherogenesis in large arteries, we investigated mitochondrial dynamics-related proteins in various areas in the mouse aorta. In the areas submitted to low shear stress (inner part of the aortic cross), OPA1 mRNA expression was reduced and DRP1 mRNA expression was increased compared to aortic areas subjected to high shear stress. These observations led to investigate atherosclerosis in *Opa1*^{+/-} and *Drp1*^{+/-} mice. These mice, when crossed with *Ldlr*^{-/-} mice and fed high fat diet, develop more atherosclerosis in *Opa1*^{+/-} and less in *Drp1*^{+/-} mice compared to WT. Thus, dis-regulation in mitochondrial dynamics proteins altered endothelium dependent functions in atherosclerotic mice, and increasing plaque formation with reduced fusion.

Resumé

Les mitochondries sont des organites dynamiques modifiant leurs membranes interne et externe par des mécanismes de fission et de fusion. En réponse aux forces de cisaillement induites par le flux sanguin, les mitochondries des cellules endothéliales (CEs) subissent en permanence des mécanismes de fusion et de fission. Nous avons émis l'hypothèse que l'altération de la dynamique mitochondriale pourrait modifier la fonction des CEs entraînant des dommages vasculaires. Pour cela, nous avons étudié le rôle de la déficience en OPA1 et DRP1 dans les CEs et l'homéostasie vasculaire en utilisant des souris déficientes en protéine de fusion OPA1 et en protéine de fission DRP1 (souris hétérozygotes *Opa1 +/-* et *Drp1 +/-*). Nous avons étudié le niveau d'expression des protéines liées à la dynamique mitochondriale dans les CEs soumises à un flux laminaire, protecteur (mis en évidence par la régulation à la baisse de eNOS et la régulation à la hausse de l'endothéline-1) et nous avons observé une réduction de l'expression de l'ARNm de la protéine de fusion OPA1 et une augmentation de l'expression de l'ARNm de la protéine de fission DRP1. Le contraire a été observé dans les CEs soumises à un flux perturbé. Après une stimulation aiguë des CEs par l'histamine, les CEs dépourvues de protéines OPA1 et DRP1 ont montré une diminution de la libération intracellulaire de Ca^{2+} par rapport aux cellules contrôles. En outre, ces cellules ont montré une capacité de migration réduite par rapport aux cellules contrôles. Comme les forces de cisaillement déterminent l'athérogenèse dans les grandes artères, nous avons étudié les protéines liées à la dynamique mitochondriale dans les diverses zones de l'aorte chez la souris. Dans les zones soumises à un faible cisaillement (partie interne de la crosse aortique), l'expression de l'ARNm d'OPA1 a été réduite et l'expression de l'ARNm de DRP1 a été augmentée par rapport aux zones aortiques soumises à un cisaillement athérogène. Ces observations ont conduit à étudier l'athérosclérose chez les souris *Opa1 +/-* et *Drp1 +/-*. Ces souris, lorsqu'elles sont croisées avec des souris *Ldlr -/-* et nourries avec un régime riche en graisses, développent plus d'athérosclérose chez *Opa1 +/-* et moins chez les souris *Drp1 +/-* par rapport aux souris WT. Ainsi, la dysrégulation des protéines de la dynamique mitochondriale a modifié les fonctions dépendantes de l'endothélium chez les souris atteintes d'athérosclérose et réduit la formation de la plaque avec une fusion réduite.

Titre : Les modifications de la dynamique mitochondriale induisent un dysfonctionnement des cellules endothéliales et une augmentation de l'athérosclérose chez la souris.

Mots clés : Endothélium vasculaire, Débit sanguin, Forces de cisaillement, Dynamique Mitochondriale, OPA1, DRP1, Athérosclérose,

Résumé: Les mitochondries sont des organites dynamiques modifiant leurs membranes par des mécanismes de fission et de fusion. En réponse aux forces de cisaillement induites par le flux sanguin, les mitochondries des cellules endothéliales (CEs) subissent en permanence des mécanismes de fusion et de fission. Nous avons émis l'hypothèse que l'altération de la dynamique mitochondriale pourrait modifier la fonction des CEs entraînant des dommages vasculaires. Pour cela, nous avons étudié le rôle de la déficience en OPA1 et DRP1 dans les CEs et l'homéostasie vasculaire en utilisant des souris hétérozygotes Opa1^{+/-} et Drp1^{+/-}. Les CEs dépourvues de protéines OPA1 et DRP1 ont montré une diminution de la libération intracellulaire de Ca²⁺ par rapport aux cellules contrôles. En outre, ces cellules ont montré une capacité de migration réduite par rapport aux

cellules contrôles. En plus, nous avons étudié les protéines liées à la dynamique mitochondriale dans les diverses zones de l'aorte. Dans les zones soumises à un faible cisaillement, l'expression de l'ARNm d'OPA1 a été réduite et l'expression de l'ARNm de DRP1 a été augmentée par rapport aux zones soumises à un cisaillement athérogène. Ces souris, lorsqu'elles sont croisées avec des souris Ldlr^{-/-} et nourries avec un régime riche en graisses, développent plus d'athérosclérose chez Opa1^{+/-} et moins chez les souris Drp1^{+/-} par rapport aux souris WT. Ainsi, la dysrégulation des protéines de la dynamique mitochondriale a modifié les fonctions dépendantes de l'endothélium chez les souris atteintes d'athérosclérose et réduit la formation de la plaque avec une fusion réduite.

Title: Altered mitochondrial dynamics induce endothelial dysfunction and increase atherosclerosis in mice

Keywords: Endothelial cells, Blood flow, Shear stress, Mitochondrial dynamics, OPA1, DRP1, Atherosclerosis.

Abstract: Mitochondria are dynamic organelles altering their inner and outer membranes through fission and fusion mechanisms. In response to shear stress, the mitochondria in endothelial cells (ECs) continuously undergo fusion and fission mechanisms. We hypothesized that alteration in mitochondrial dynamics could alter ECs function leading to vascular damages. For that, we investigated the role of OPA1 and DRP1 deficiency in ECs and vascular homeostasis using mice lacking fusion protein OPA1 and fission protein DRP1 (heterozygous Opa1^{+/-} & Drp1^{+/-} mice). Cells lacking OPA1 and DRP1 protein have shown a decreased intracellular Ca²⁺ release, compared to control cells. Besides, these cells showed a decreased capability of migration compared to control cells.

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