UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea in Biologia

ELABORATO DI LAUREA

THE ROLE OF MITOCHONDRIAL DYNAMICS IN CARDIOVASCULAR DISEASES

Tutor: Dr.ssa Martina Semenzato
Istituto Veneto di Medicina Molecolare
Dipartimento di Biologia

Laureanda: Carlotta Bergamin

ANNO ACCADEMICO 2024/2025

“Study hard what interests you the most in the most undisciplined,
irreverent and original manner possible”
— Richard Feynman

ABSTRACT

Cardiovascular diseases are among the leading causes of death worldwide nowadays (World Health Organization, 2021). They represent a group of disorders that affect the heart and the vascular system, characterised by the death of myocytes, i.e. the specialised cells of the cardiovascular system, which leads to permanent tissue damage and, potentially, the death of the organism. On a molecular level, the onset of several cardiovascular disorders might be linked to mitochondrial dysfunction. These organelles are responsible for ATP production, calcium homeostasis, and apoptosis, all of which are essential for cell survival (Quintana-Cabrera & Scorrano, 2023). Moreover, mitochondria regulate their shape, size, and number through mitochondrial dynamics. These dynamic equilibrium is a balance between fission and fusion, which maintains mitochondrial structure (Yu et al., 2020) and allows the correct organelle function and the overall cellular health. Imbalanced mitochondrial dynamics play a role in the development of cardiovascular diseases: therefore, studying mitochondria-shaping proteins is essential to understand this connection and to develop therapeutic strategies to restore a proper mitochondrial function.

Table of Contents

ABBREVIATIONS

CVD	Cardiovascular disease
IMM	Inner mitochondrial membrane
OMM	Outer mitochondrial membrane
IMS	Intermembrane space
ATP	Adenosine triphosphate
GTP	Guanosine triphosphate
mtDNA	Mitochondrial DNA
TM	Transmembrane domain
Fzo1	Fuzzy onion 1
Mgm1p	Mitochondrial genome maintenance 1
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
ER	Endoplasmic reticulum
Opa1	Optic atrophy 1
CMT2A	Charcot-Marie-Tooth disease type 2A
Pgc-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
Err-α	Estrogen-related receptor-alpha
OMA1	M-AAA protease 1
YME1L1	YME1 Like 1 ATPase
PARL	Presenilin associated rhomboid like
Dnm1	Dynamin related protein 1
Drp1	Dynamin-related protein 1
Fis1	Fission factor 1
Mff	Mitochondrial fission factor
EGFP	Enhanced green fluorescent protein
Tg	Transgenic
OS-PCM	Organelle-specific phase contrast microscopy
CNN	Convolutional neural network
IRI	Ischemia/reperfusion injury
ROS	Reactive oxygen species
mPTP	Mitochondrial permeability transition pore
DKO	Double knockout
KO	Knock out
WT	Wild type
ZDF	Zucker diabetic fat rats
ZL	Zucker lean rats
HG	High glucose
NRCMs	Neonatal rat cardiomyocytes
Orai1	Ca^{2+} release-activated calcium channel protein 1
LV	Left ventricular
ERK	Extracellular signal-regulated kinase

CnA	Calmodulin-binding catalytic subunit A
FMD	Flow-mediated dilation
ECs	Endothelial cells
PAH	Pulmonary artery hypertension
PASMCs	Pulmonary artery smooth muscle cells
CDK1	Cyclin-dependent kinase 1
HIF-1α	Hypoxia-inducible factor 1α
IDCM	Idiopathic dilated cardiomyopathy
LVEF	Left ventricular ejection fraction
NRVMs	Neonatal rat ventricular myocytes
β-AR	β-adrenergic receptor
miRNA	Micro RNA
HO-1	Heme oxygenase 1

1. INTRODUCTION

One of the major causes of death worldwide is cardiovascular disease (CVD), a group of disorders that affect the heart and the blood vascular system. These diseases may lead to permanent tissue damage and even death. In 2019, 17.9 million people died from CVDs (World Health Organization, 2021).

The onset of several cardiovascular disorders has been linked to mitochondrial dysfunction. Mitochondria are responsible for ATP production, calcium homeostasis, and apoptosis, key processes required for cell survival (Quintana-Cabrera & Scorrano, 2023). Additionally, mitochondria regulate their shape, size, and number through mitochondrial dynamics (Yu et al., 2020) a process involving a balance between fission and fusion. This allows to maintain mitochondrial structure and in turn the correct functioning of these organelles. Consequently, safeguards the physiological state of the cells. Imbalanced mitochondrial dynamics play a role in the development of cardiovascular diseases. Therefore, studying mitochondria-shaping proteins is essential to understand this connection and develop therapeutic methods that target these proteins to re-establish correct mitochondrial function.

The main objectives of this thesis are to describe the proteins and mechanisms of mitochondrial dynamics, with the aim of understanding the connection between the fission and fusion processes and the development of cardiovascular diseases.

2. SHAPE AND FUNCTION OF MITOCHONDRIA ARE INTERCONNECTED

Mitochondria are membrane-bound organelles, found in most eukaryotic cells, with two membranes, an intermembrane space and a matrix (Yu et al., 2020). The external structure of this organelle consists of an outer mitochondrial membrane (OMM), which is a phospholipid bilayer with several transmembrane proteins, allowing the exchange of materials with the cytoplasm and other organelles or membranes. On the other hand, the inner mitochondrial membrane (IMM) represents a more selective barrier, so only some molecules can pass. The inner space of the mitochondria is characterized by the matrix and the cristae, which are invaginations of the inner membrane. The cristae are fundamental structures for the metabolic activity of mitochondria because the protein complexes involved in cellular respiration, including the four oxidative phosphorylation complexes and the ATP synthase, are anchored to the cristae. The intermembrane space (IMS) is

located between the outer and inner membranes. This organelle is responsible for producing the chemical energy source of the cell, i.e. adenosine triphosphate (ATP), through oxidative phosphorylation, and this is why mitochondria are called the powerhouses of the cell. It also has other functions, such as regulating the cell cycle and cell proliferation, mitophagy, autophagy, and cell apoptosis. Additionally, it regulates the calcium signalling and the detoxification of reactive oxygen species (Quintana-Cabrera & Scorrano, 2023; Yu et al., 2020).

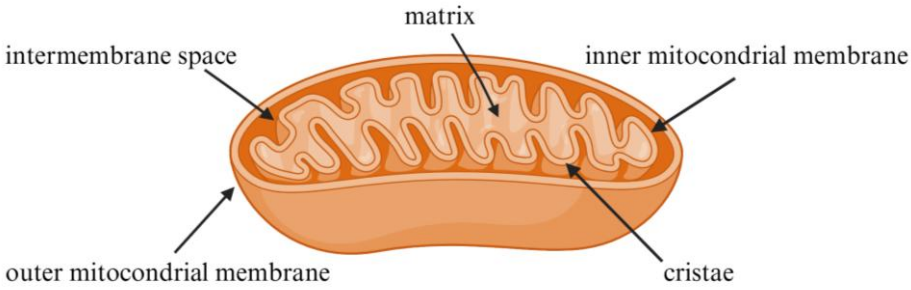

Figure 1. Schematic representation of a mitochondrion (created with BioRender Software)

3. MITOCHONDRIAL DYNAMICS

Mitochondria can perform their functions when a normal structure is maintained. Thus, it is important to understand which processes can regulate their morphology. Mitochondria are defined as highly dynamic organelles due to their ability to change their shape in response to changing environmental conditions and accordingly to the physiological state of the cell (Quintana-Cabrera & Scorrano, 2023; Yu et al., 2020). Mitochondrial dynamics regulate changes in mitochondrial shape, and it is defined as a balance between fission and fusion. These two mechanisms regulate the size, number, distribution, quality control, and transport of mitochondria within cells (Yu et al., 2020). Fusion and fission can occur through the action and interactions of several proteins that have been discovered to be responsible for mitochondrial dynamics. The so-called mitochondrial-shaping proteins have been studied for several years in yeast, and their homologs have also been identified in mammals (Quintana-Cabrera & Scorrano, 2023). Even though the proteins involved in the fission process and those responsible for the fusion have different functions, some share common

structures because their activity is associated with a GTPase domain. For example, the mammalian proteins Mfn1 and 2, Opa1, and Drp1 are part of the dynamin-related protein family since they all share a GTPase domain, which hydrolyses guanosine triphosphate (GTP) to activate the proteins and regulate the mitochondrial shape.

3.2 MITOCHONDRIAL FUSION

Fusion is "the process of merging two or more mitochondria to generate a larger organelle" (Quintana-Cabrera & Scorrano, 2023). This is essential for exchanging mitochondrial DNA (mtDNA) and proteins, rescuing damaged organelles, and is useful to increase ATP production when needed.

3.2.1 FUSION MACHINERY IN YEAST

Fusion has been studied for many years in yeast. Three proteins, Fzo1p, Mgm1p and Ugo1p, have been identified as playing a key role during this process (Yu et al., 2020).

Fzo1p

This molecule was first described in 1997 in *Drosophila melanogaster*, particularly the effects of the altered *fuzzy onion* (*Fzo1*) gene expression in the spermatids. Indeed, when the gene was absent in these cells, the giant mitochondrion, usually found in the spermatid tail, was substituted by smaller mitochondria, emphasising the role of Fzo1p as a pro-fusion protein (Quintana-Cabrera & Scorrano, 2023). It is a large dynamin-related protein with a GTPase domain, an essential structure that hydrolyses GTP to carry out the fusion process. Fzo1p is anchored to the outer mitochondrial membrane via two adjacent transmembrane domains (TM) found in the C-terminus. On the other hand, the N-terminal portion has a GTPase domain, and it faces the cytoplasm. For its location, it is understandable that this protein is involved in the fusion of the OMM (Yu et al., 2020).

Mgm1p

Mitochondrial genome maintenance 1 (Mgm1p) is a dynamin-related protein with a TM domain that binds to the inner mitochondrial membrane and a GTPase domain, found at the N-terminus. It is also characterized by two hydrophobic domains which interact with the intermembrane space and are located at the carboxyl end. This protein is essential for merging both inner and outer mitochondrial membranes, and the lack of this molecule causes mitochondrial fragmentation and genome loss, like the effect of Fzo1p absence in *D. melanogaster* spermatids (Yu et al., 2020).

Ugo1p

It has three TM domains that anchor the protein to the OMM. The amino terminal faces the cytosol and binds with Fzo1p, whereas the C-terminal in the intermembrane space interacts with Mgm1p. Therefore, "Ugo1p acts as a molecular bridge between Fzo1p and Mgm1p in mitochondrial fusion" (Yu et al., 2020) and it is essential for both inner and outer mitochondrial membrane fusion. Similar to the other pro-fusion molecules, the loss of Ugo1p leads to the unregulated division of mitochondria (Yu et al., 2020).

3.2. FUSION MACHINERY IN MAMMALS

The mitochondria-shaping proteins of yeast have homologues in mammalian cells. Mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) are homologues of Fzo1p, whereas optic atrophy 1 (Opa1) is the homologue of Mgm1. These three mammalian proteins are dynamin-related GTPases, and together they play an essential role during the three-step fusion process [Figure 3A]. This process consists of building a docking ring structure around the contact point of the outer membranes of two mitochondria to tether the organelles. After that, the hydrolysis of GTP allows the membranes to fuse due to the action of mitofusins 1 and 2, which is followed by the merging of the mitochondrial inner membranes, regulated by Opa1 (Yu et al., 2020).

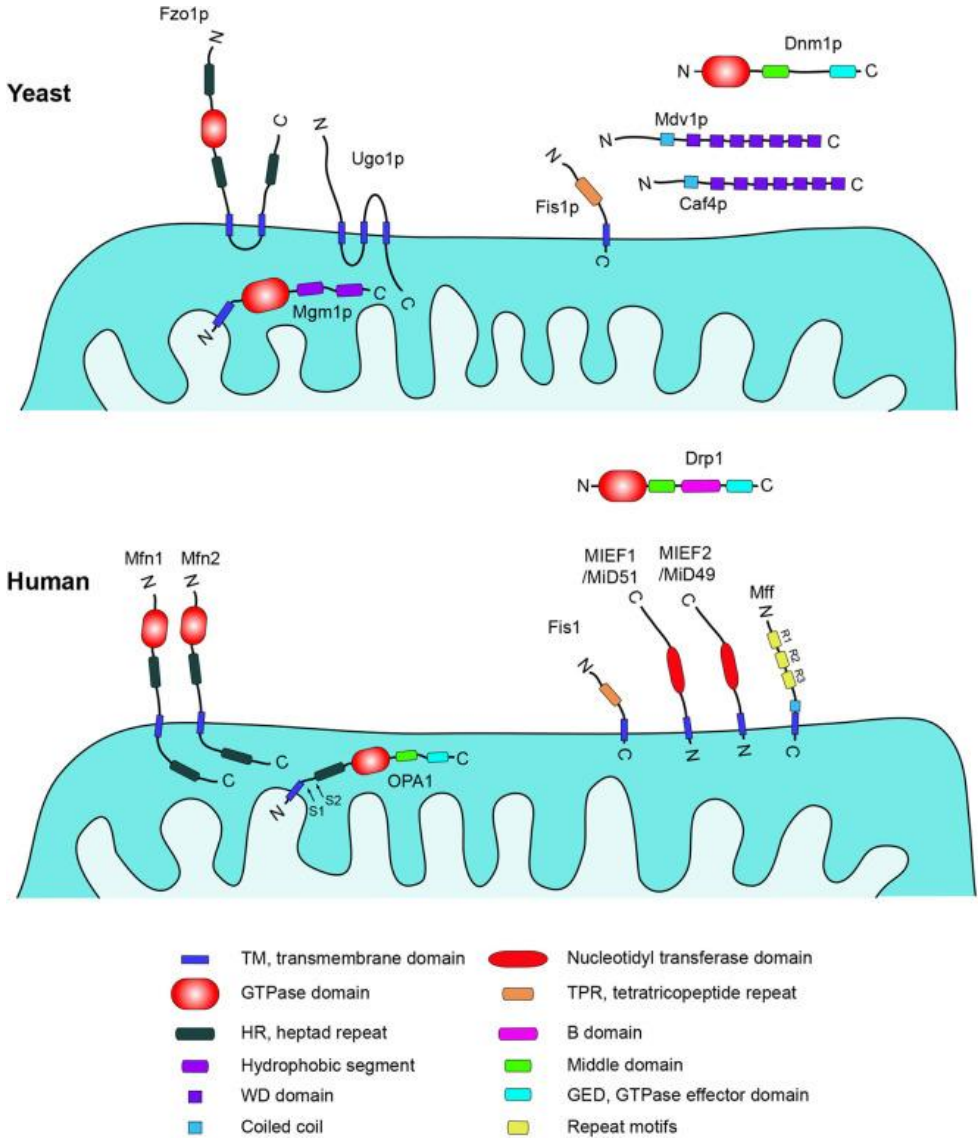

Figure 2. Schematic representation of key mitochondria-shaping proteins in yeast and human cells (Yu et al., 2020).

Mfn1 and Mfn2

Mfn1 and 2 are highly conserved among mammals. Both proteins are ubiquitously expressed, although having a high expression in highly energy demand tissues, such as skeletal muscle and heart (Quintana-Cabrera & Scorrano, 2023).

Their structure is characterized by a GTPase domain at the N-terminus, followed by two coiled-coil domains (HR1 and HR2). In between the two HR portions, there is a TM domain to interact with the OMM [Figure 2]. These proteins can accumulate at the contact site between two mitochondria and form stable homodimers or heterodimers through their GTPase domains in *trans*. Additionally, both mitofusins can create homo- and hetero-oligomers, and with

these conformations, tether the outer membranes of two neighbouring mitochondria (Yu et al., 2020). These structures and the GTP hydrolysis are the main factors that cause membrane tethering and fusion of two adjacent OMMs. In recent studies, it was also observed that HR1 can also play a role in merging the two membranes in *trans*. Mfn1 seems to have a higher GTPase activity, whereas Mfn2 is essential for endoplasmic reticulum (ER)-mitochondria interactions, which are important for calcium transport and trafficking of different phospholipids. Moreover, the two mitofusins are proven to have distinct functions because only mutations in *Mfn2* cause a type of peripheral neuropathy called Charcot-Marie-Tooth disease type 2A (CMT2A) (Yu et al., 2020). On the other hand, *Mfn2* deficiency can be compensated by Mfn1 but not by wild-type Mfn2, via hetero-oligomer formation between Mfn1 and Mfn2. Additionally, Mfn2-deficient cells have short and rod-shaped mitochondria, in contrast to the spherical ones found in cells with Mfn1 mutations (Quintana-Cabrera & Scorrano, 2023). Thus, the two proteins "are functionally correlated but non-redundant" (Yu et al., 2020). The regulation of the expression and production of mitochondria-shaping proteins is fundamental to ensure their correct mechanism of action. For example, the overexpression of Mfn1 or Mfn2 leads to perinuclear aggregations of elongated mitochondria (i.e. hypertubulation). Thus, many factors regulate Mfn1 and 2 transcription and control post-transcription and translation modifications. The proteins involved in the transcription regulation are, for example, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc-1α) and beta (Pgc-1β), and estrogen-related receptor-alpha (Err-α). Several microRNAs (miRNAs) are involved in downregulating Mfn1 and 2. Moreover, the two proteins can undergo post-translational modifications such as oxidation, ubiquitylation, and phosphorylation, which can either inhibit or stimulate their action (Quintana-Cabrera & Scorrano, 2023).

Opa1

Opa1 is the homolog of the yeast Mgm1p, and it was discovered by studying a gene mutation causing optic atrophy, an autosomal dominant disease. It is a dynamic-related protein with an amino end that carries a TM domain, for IMM binding, and an HR domain. It also possesses a GTPase domain, a middle domain, and a GTPase effector domain, near the carboxyl end (Yu et al., 2020). Opa1 is essential for the fusion of the two adjacent inner mitochondrial membranes, and in humans, it has been discovered that eight different variants of mRNA exist through alternative splicing. It can be found in two different forms: l-Opa1, a long and membrane-anchored form, and a short and soluble one (s-Opa1) (Yu et al.,

2020). The second form is produced from the l-Opa1 by the action of two metalloproteases, M-AAA protease 1 (OMA1) and YME1 Like 1 ATPase (YME1L1), that can cleave the protein at the S1 and S2 sites [Figure 2] to originate the short form. The processing of l-Opa1 can also be done by presenilin associated rhomboid like (PARL) protease, generating an IMS soluble fraction that binds with l-Opa1 to ensure cristae junctions. Opa1 can also undergo other modifications, such as O-GlcNAcetylation and ubiquitination (Quintana-Cabrera & Scorrano, 2023).

The fusion of the two neighbouring IMMs requires that the Opa1 of one mitochondrion binds in *trans* with a phospholipid called cardiolipin, located in the IMM of the other organelle. Additionally, the interaction between two l-Opa1 in *trans* can occur and optimise the fusion process. On the other hand, the binding between two s-Opa1 can only guarantee tethering of the IMM but not the fusion. Surprisingly, an accumulation of the short form can induce fission in *Yme1l*-deficient cells, thus "the l-/s-Opa1 ratio is critical for fusion", and both forms "must cooperate to increase the poor fusion efficiency of either form alone" (Quintana-Cabrera & Scorrano, 2023).

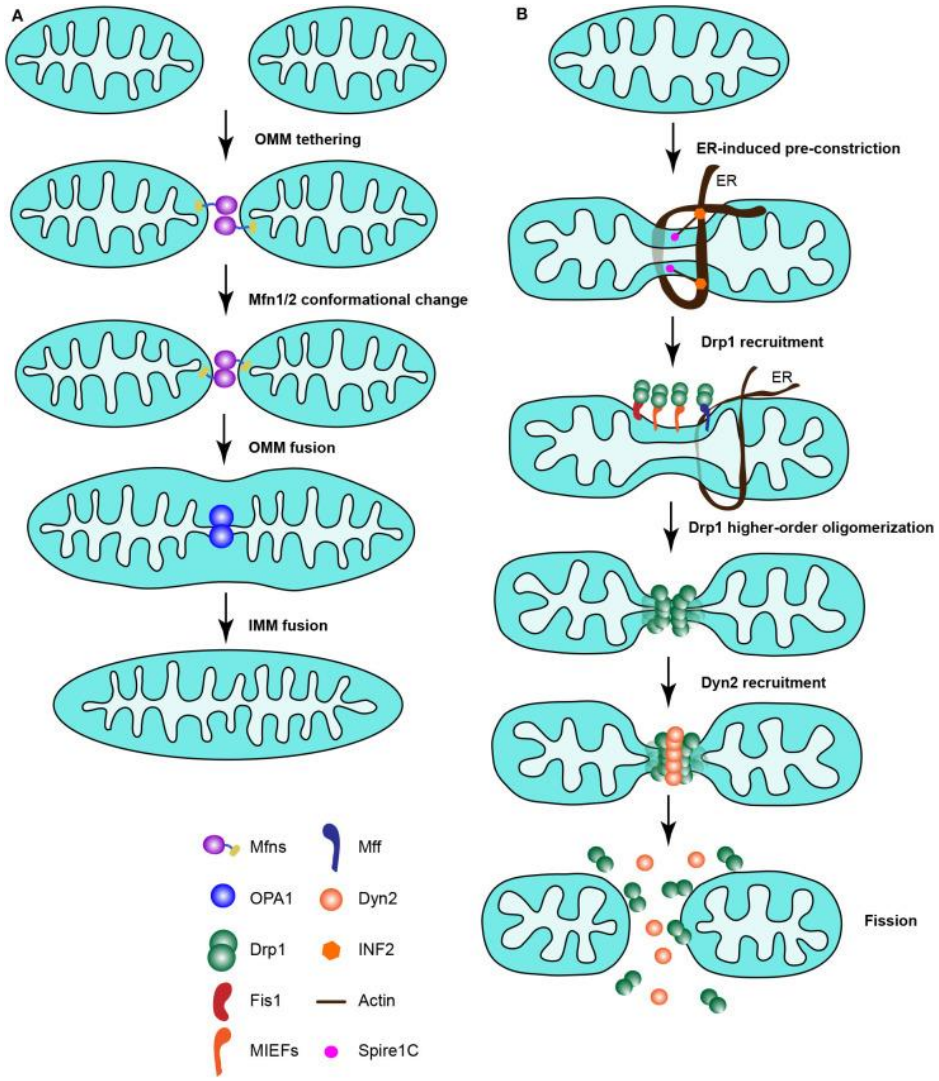

Figure 3. Mitochondrial dynamics: schematic representation of (A) fusion and (B) fission (Yu et al., 2020).

3.3 MITOCHONDRIAL FISSION

Fission is the process of generating smaller mitochondria from a single bigger organelle. It regulates mitochondrial and cellular homeostasis by sorting organelles with low membrane potential or damaged mtDNA to mitophagy. During mitosis, fission allows the distribution of mtDNA and old mitochondria to daughter cells and is essential for scattering old and young mitochondria to maintain the ability of cells to differentiate (Quintana-Cabrera & Scorrano, 2023). Moreover, fission activity is very high during cancer cell proliferation and invasion, and it is crucial for cell renewal and resistance to differentiation in some types of stem cells (Guo et al., 2021).

Similarly to the fusion process, fission requires the action of mitochondria-shaping proteins.

3.3.1 FISSION MACHINERY IN YEAST

The key yeast proteins involved in the fission process are Dnm1p, Fis1p, Mdv1p, and Caf4p [Figure 2]. Dynamin related protein 1 (*Dnm1*) was the first fission gene that was discovered by studying yeast mutants with altered mitochondrial shape. Dnm1p and the homolog Drp1 of mammals are the central regulators of fission, and the other fission proteins have the role of recruiting Dnm1p or Drp1 to the mitochondrial membrane, so they are defined as protein adaptors or receptors (Quintana-Cabrera & Scorrano, 2023; Yu et al., 2020).

Dnm1p

Dnm1p is a dynamin-related protein with a GTPase domain at the N-terminal, a middle domain and a GTPase effector domain. It is usually found in the cytosol, and it is recruited to the mitochondrial membrane by Mdv1p or Caf4p, which function as molecular bridges to connect Dnmp1 with Fis1p. This is the first step of mitochondrial fission, and after this recruitment, Dnm1p self-assembles around the mitochondrial constriction site in a ring-like structure by forming oligomers. Finally, the fission can occur. The importance of this protein during fission can be demonstrated by the fact that Dnm1p-null cells show long tubular networks of mitochondria and have mitochondrial membranes collapsed to one side of the cell (Yu et al., 2020).

Fis1p

Fis1p is the fission protein that directly interacts with the outer mitochondrial membrane via the C-terminal TM domain. On the other hand, the N-terminal carries a tetratricopeptide repeat (TPR) that faces the cytosol. This portion forms a concave surface, and it also has a short helix, which is essential for binding and recruiting Mdv1p or its paralog Caf4p to the concave surface. When Fis1p is deleted in the cells, a mitochondrial reticular formation occurs, proving that this protein is essential for the fission process (Yu et al., 2020).

Mdv1p and Caf4p

These two proteins are soluble in the cytosol. Their structure is characterised by a middle coiled-coil domain, a WD repeat domain at the C-terminus [Figure 2]. Due to this molecular morphology, both Mdv1p and Caf4p can work as protein adaptors and bridges by connecting Dnm1p and Fis1p through the interaction between their WD repeat and the C-terminal of Dnm1p, and the association of their N-terminal extension with the Fis1p N-terminal portion (Yu et al., 2020). Mdv1p and Caf4p are paralogs although Mdv1p is more active than Caf4p in promoting fission. Caf4p but not Mdv1p in association with Fis1p can determine the polarized localization of Dnm1p clusters on the mitochondrial surface. When there is a deletion of one of the two genes, yeast cells' mitochondria maintain a normal morphology; however, if both genes are not expressed, the organelles become elongated and assume a net-like shape. The overexpression of either of the two proteins seems to have an inhibitory effect on fission, probably because the overexpression of Mdv1p or Caf4p can block the recruitment of Dnm1p to mitochondria (Yu et al., 2020).

3.3.2 FISSION MACHINERY IN MAMMALS

The fission process in mammals is more complex than in yeast. It requires a dynamin-related GTPase, Drp1, which can be recruited to the mitochondrial surface by either one or more of these mitochondrial receptors: Fis1, Mff, MiD49 and MiD51. It is still not clear why these protein receptors simultaneously exist in the cell and whether they work independently or in a coordinated way (Yu et al., 2020).

Drp1

Dynamin-related protein 1 is the ortholog of yeast Dnm1p. It has an N-terminal GTPase domain, a dynamin-like middle domain, a variable domain (B domain) and a GTPase effector domain (GED) located at the C-terminus [Figure 2]. First, Drp1 is recruited by the protein adaptors to the mitochondrial membranes, which are marked and constricted by the ER membrane extensions (Yu et al., 2020). Another element that is associated with the ER and the mitochondrial membranes is the actin cytoskeleton. This structure interacts with the ER via inverted formin 2 (INF2) and binds to the mitochondrial surface through a promoter of the actin assembly, called actin-nucleating protein Spire1C. INF2 and Ca^{2+} induce actin polymerization at the ER-mitochondria contact site. This process is essential for IMM fission, and it promotes Drp1 recruitment. Then, Drp1 proteins from

multimers at the fission sites, and finally, GTP is hydrolysed by the GTPase domain to sever mitochondria. The coordination of all the components and the interplay between the receptors is still not fully understood (Yu et al., 2020). It seems that the final scission is carried out by Dynamin 2 (Dyn2), another dynamin-related protein, responsible for membrane remodelling of several organelles. However, recent studies suggest that it is not essential during mitochondrial fission since its ablation does not inhibit fission. The activity of Drp1 is regulated by transcriptional and post-translational modifications. The transcription is regulated by non-coding RNAs, while multiple processes allow post-translational modifications such as phosphorylation, ubiquitylation, SUMOylation, O-GlcNAcylation, and nitrosylation (Quintana-Cabrera & Scorrano, 2023).

Fis1

Fission protein 1 was the first adaptor protein identified in mammals. The molecular structure is very similar to Fis1p. It has a C-terminal TM domain that anchors the protein to the OMM, whereas the N-terminal faces the cytosol, and it carries a TPR-like core domain, important for Drp1 recruitment. It seems that Fis1 facilitate the fission process in specific cell types and under particular conditions, such as mitophagy or apoptosis. The protein can still recruit Drp1 under physiological conditions, but the interaction between Fis1 and Drp1 is stronger when cells are under stress (Yu et al., 2020). Additionally, its overexpression causes mitochondrial fragmentation, probably because Fis1 can interact with the pro-fusion proteins (Mfn1, Mfn2, and Opa1) and block their GTPase activity. On the other hand, the gene knockdown results in mitochondrial elongation in HeLa cells and *Fis1*-null mouse embryonic fibroblasts. It is the homolog of yeast Fis1p, although growing evidence shows that their function has diverged. As a matter of fact, “human Fis1 cannot rescue the mutant phenotype in yeast cells lacking Fis1p, while yeast Fis1p, when expressed in human HeLa cells, is targeted to mitochondria, but does not affect mitochondrial morphology” (Yu et al., 2020). This demonstrates that mammalian Fis1 and yeast Fis1p have diverged functions, although maintaining a similar morphology.

Mff

Mitochondrial fission factor is the most important adaptor for Drp1 recruitment, and the best studied so far. It was discovered via a small interfering RNA (siRNA) screen in *Drosophila melanogaster*. It has not been identified in yeast, since the adaptor proteins Mdv1p and Caf4p do not have homologs in mammals (Yu et al.,

2020). Mff is characterized by a C-terminal TM domain, which interacts with the OMM. The N-terminus, facing the cytosol, have three short amino acid repeats (R1, R2, and R3 motifs) and a coiled-coil domain [Figure 2]. The region containing the R1 and R2 motifs is crucial for Drp1 recruitment. The gene of Mff can be transcribed into nine different variants via alternative splicing, and if the gene expression is altered (i.e. a knockdown event), the recruitment of Drp1 and the fission are both compromised. Mff can undergo phosphorylation by protein kinase AMP-activated (AMPK) to enhance its pro-fission action, and this occurs when increased levels of AMP in the cell are detected, a sign of mitochondrial malfunction. Mff recruits oligomeric and active forms of Drp1 at the mitochondrial midzone, in contrast to MiD49 and 51 interact with Drp1 to allow its oligomerization (Quintana-Cabrera & Scorrano, 2023; Yu et al., 2020).

MiD49 and MiD51

MiD49/MIEF2 and MiD51/MIEF1 are paralogs and recently discovered adaptors that have the same function as Fis1 and Mff, to recruit Drp1 to the mitochondrial surface. They are highly conserved in vertebrates, while they have not been identified in plants and invertebrates. Both proteins have a TM domain at the N-terminal to interact with the OMM and a nucleotidyl transferase domain [Figure 2], but with different activities. In MiD51, the domain can bind nucleotide diphosphate molecules, such as ADP and GDP, whereas the domain in MiD49 does not have this function. Moreover, ADP-MiD51 interaction can promote Drp1 oligomerization and activation (Yu et al., 2020). The two adaptors can be found in monomeric and dimeric structures (both homo- and heterodimeric forms). Additionally, biochemical analysis shows that MiD51 mostly appears as dimers, while MiD49 appears as oligomers. Regarding their expression, if either MiD is overexpressed, the effect is like Fis1 overexpression: extensive recruitment of Drp1 to the mitochondrial surface and a subsequent increase in fission. On the other hand, it was interesting to observe that the overexpression of both adaptors leads to mitochondrial elongation rather than fragmentation, probably due to a sequestration of surface and an inhibition of Drp1 GTPase activity (Yu et al., 2020) .

3.4 FUSION-FISSION BALANCE IS CRUCIAL FOR MAINTAINING MITOCHONDRIAL HEALTH

Mitochondrial dynamics are crucial in both normal and pathological conditions. A balance between fusion and fission is crucial to maintain a correct morphology and a proper functioning of these organelles. Thus, multiple studies suggest a crosstalk between fusion and fission machineries. For example, the fission protein Fis1 can recruit Drp1 to the mitochondrial membrane for fission, and it can also promote this process by binding mitofusins or Opa1 to inhibit their pro-fusion activity, showing that Fis1 regulates both machineries because it can both facilitate the fission and/or block the fusion (Yu et al., 2020). Moreover, the processing of Opa1 is involved in the regulation of both machineries. Indeed, the short forms of this protein seem to promote mitochondrial fragmentation, but they are also crucial during fusion. It has been shown that whichever role s-Opa1 is given, it depends on the ratio between this structure and l-Opa1. When there is an excessive production of the short molecule, the balance is shifted towards mitochondrial fragmentation. Another evidence of the crosstalk between the two machineries is given by the possible interaction that can be established between Drp1 and Mfn2. Moreover, Drp1 can contribute to mitochondrial fusion in Mfn1- or Mfn2-deficient cells (Yu et al., 2020).

4. MITOCHONDRIAL DYNAMICS AND CARDIOVASCULAR DISEASES

The interplay between mitochondrial dynamics consists of continuous cycles of fission and fusion to maintain mitochondrial and cellular homeostasis (Hall et al., 2016). Indeed, there is a connection between unbalanced mitochondrial dynamics, causing mitochondrial dysfunction, and the development of several disorders, such as cardiovascular and neurodegenerative diseases, i.e. Alzheimer's disease and Parkinson's syndrome, and even cancer.

The heart is a metabolically demanding organ that depends critically on mitochondrial activity (Wai et al., 2015). At the same time, mitochondrial activity is deeply connected to mitochondrial dynamics balance because fission and fusion processes determine the shape of these organelles, and the proper morphology ensures the correct mitochondrial functioning.

4.1 Acute myocardial infarction

Acute myocardial infarction or heart attack is a disease that is caused by an insufficient blood supply (i.e. ischemia) to the heart, which is a vital organ. One solution to reduce infarct size and damage is represented by blood reperfusion, right after the ischemic event. However, reperfusion can cause *per se* injuries in the myocardium through a compromised mitochondrial function. Ischemia/reperfusion injury (IRI) induces an overload of Ca^{2+} in the organelle, the overproduction of reactive oxygen species (ROS), and the opening of the mitochondrial permeability transition pore (mPTP). These three conditions cause mitochondrial dysfunction, leading to cardiomyocyte death (Hall et al., 2016).
Mitochondria tend to increase the fission activity of Drp1 during IRI. Consequently, the inhibition of Drp1 can protect heart tissue from I/R injury and reduce infarct size. On the other hand, a study by Hall et al. (2016) suggested the potential benefits of ablating the pro-fusion proteins Mfn1 and Mfn2 to minimise the effects of IRI. Firstly, they performed a double knockout (DKO) of both mitofusins in adult mice cardiomyocytes by administering tamoxifen to mice. The mitochondria of DKO mice appeared fragmented, exhibited a compromised cristae structure, and a reduction in maximal respiration. Moreover, the hearts of Mfn1/Mfn2 DKO mice had impaired contractile function compared to those of the wild type (WT). Upon IRI, DKO mice showed impaired mitochondrial respiration in comparison with WT mitochondria. Additionally, DKO mitochondria had a depleted overload of Ca^{2+} because of the absence of a connection between the sarcoplasmic reticulum and the mitochondria, which allows calcium intake. Indeed, Mfn2 can regulate this interaction, and since the protein was ablated, calcium transfer was inhibited. As a result, DKO mitochondria were subjected to less oxidative stress, considering that Ca^{2+} overload can trigger this kind of stress in mitochondria. The opening of the mitochondrial permeability transition pore (mPTP) was evaluated in both WT and DKO mitochondria because the opening of the mPTP facilitates the entrance of calcium into the organelle, promoting swelling. After ischemia/reperfusion injury, there was a resistance to mPTP opening in DKO, compared to the WT.
Thus, ablation of both Mfn1 and Mfn2 results in protection of the heart from the detrimental effects of IRI treatment can give. The DKO hearts have a reduction in infarct size when compared to the WT ones, meaning that the ablation can protect against acute myocardial infarction. These results are very promising, even though it is important to consider the deleterious effects of a permanent ablation of Mfn1 and Mfn2, key proteins of the fusion process. For example, this research

demonstrated an impairment of respiratory capacity in the DKO mitochondria, which can cause damage in the long run. Thus, only a transient inhibition of mitofusins can be propose as a possible strategy for cardioprotection (Hall et al., 2016).

4.2 Heart failure

Heart failure is a pathological condition characterised by necrosis, fibrotic scarring, and ventricular remodelling. From a metabolic point of view, this cardiac disease is characterized by a switch from fatty acid oxidation to the utilisation of glucose as the primary energy source (Wai et al., 2015). All these conditions lead to the development of dilated cardiomyopathy. Wai et al. (2015) observed that the processing of Opa1 is crucial for maintaining the metabolic activity required by the heart, a highly energy-demanding organ.

Opa1 exists in two forms: a long form (l-Opa1) and a short one (s-Opa1). To obtain the second form, l-Opa1 undergoes proteolytic cleavage, regulated by the action of the peptidases OMA1 and YME1L. In their experiments, *Yme1l*$^{-/-}$ embryos displayed a generalised developmental delay, and their isolated hearts did not function properly, showing that the YME1L enzyme is essential for embryonic development. *Yme1l* knock-out (KO) mice exhibited a shorter life span and weight loss right before their passing. From longitudinal echocardiographic analyses, they demonstrated a progressive mitochondrial dysfunction associated with the onset of dilated cardiomyopathy (i.e. necrosis, fibrosis, and dilated left ventricular chamber), and eventually heart failure. An additional indicator of the pathogenic state was the metabolic switching from lipid oxidation to carbohydrate utilization, which was shown by in vivo (PET-CT to measure uptake of fluorodeoxyglucose) and in vitro (monitoring of glycolysis rates and glucose levels with gas chromatography-mass spectrometry) experiments that measured an increase in glucose uptake in the *Yme1l*-deficient cardiomyocytes. Moreover, mitochondria appeared smaller in adult mice isolated cardiomyocytes lacking YME1L, indicating impaired mitochondrial dynamics. This is because the lack of YME1L causes stress and dysfunction in mitochondria, and both conditions induce the activation of the protease OMA1, which increases the production of s-Opa1. The accumulation of the short form leads to mitochondrial fragmentation; thus, the absence of YME1L is associated with imbalanced mitochondrial dynamics, which eventually results in cardiomyocyte death. The study shows that mitochondrial morphology can be rescued when OMA1 is inactivated by gene

deletion in mouse embryonic fibroblasts lacking YME1L. A similar result was observed for DKO mice lacking both OMA1 and YME1L in cardiomyocytes. Indeed, DKO hearts had normal cardiac function, there was no sign of fibrosis, and fragmentation was inhibited. Interestingly, mice lacking YME1L in both cardiomyocytes and skeletal muscle did not have a shorter life span but presented smaller mitochondria, meaning that the Opa1 processing affected their morphology, just like in *Yme1l*-deficient hearts. Additionally, there was an impairment in the glucose metabolism of muscle cells, which could be restored by *Oma1* ablation. Thus, the regulation of mitochondrial morphology is deeply connected to metabolic activity, and by changing the metabolism, a normal cardiac function is restored when *Yme1l*-deficient mice are fed a high-fat diet. Although mitochondria still had abnormal morphologies since the high-fat diet had not rescued the proteolytic activity of YME1L or OMA1-dependent, stress-induced processing of Opa1, the metabolic switch helped the suppression of heart failure.

In summary, the research claims that YME1L is essential for embryonic survival. The processing of Opa1 needs to be regulated for balanced mitochondrial dynamics, even if the proteolytic cleavage of Opa1 is dispensable, since mice lacking both YME1L and OMA1 had normal cardiac function. The absence of YME1L triggers the activation of OMA1, which is responsible for s-Opa1 accumulation, causing increased mitochondrial fission. *Oma1* ablation can rescue mitochondrial morphology and cardiac activity in *Yme1l*-deficient cardiomyocytes. Moreover, high-fat dieting restores normal cardiac function in mice, but not mitochondrial morphology, and this could be a potential treatment for cardiomyopathy (Wai et al., 2015).

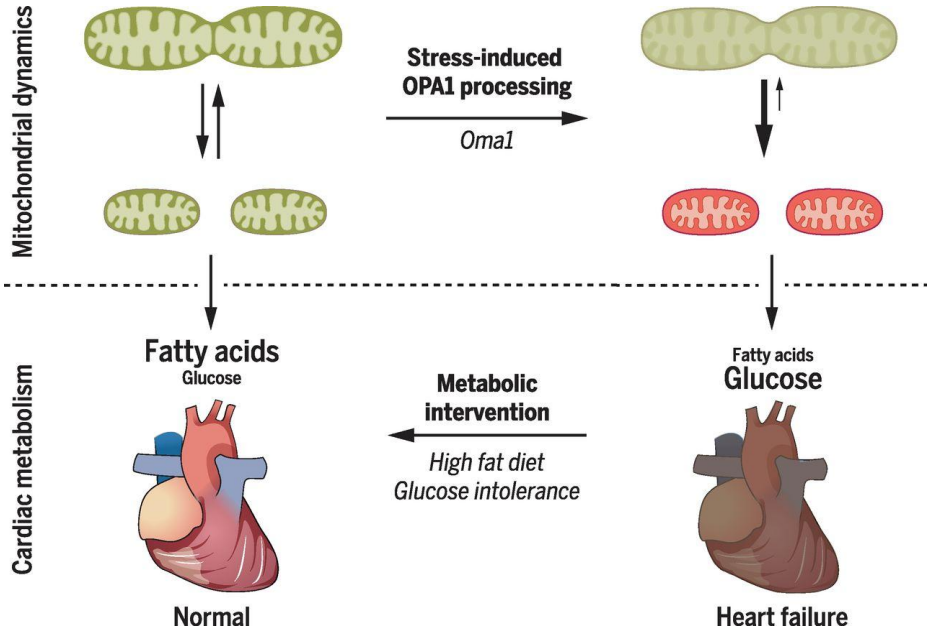

Figure 6. Normal cardiac conditions are associated with balanced mitochondrial dynamics, while an increase in mitochondrial fission, caused by OMA1 overexpression and s-Opa1 accumulation, leads to a shift to glucose metabolism and heart failure (Wai et al., 2015).

4.3 Diabetic cardiomyopathy

Diabetic cardiomyopathy is a myocardial dysfunction characterized by left ventricular (LV) hypertrophy and impaired cardiac activity. It is a major cardiovascular complication of diabetes mellitus, and its pathogenesis involves metabolic disorders, mitochondrial dysfunction, impaired cardiomyocyte calcium handling, and inflammation (Wu et al., 2021). To understand the connection between mitochondrial dysfunction and type 2 diabetes, a research study by Wu et al. (2021) tested twenty rats and six mice. Ten male Zucker diabetic fat (ZDF) rats were fed a high–fat diet, whereas the ten male Zucker lean (ZL) rats ate a normal diet. The diabetic rats had increased blood glucose and higher body weight compared to the ZL rats. In ZDF rats, the size of cardiomyocytes had increased, and many signs of cardiac hypertrophy were detected, such as an increase in "LV end diastolic anterior wall thickness, LV wall end diastole, left ventricular ejection fraction, and left ventricular fractional shortening" (Wu et al., 2021).

In this study, they aimed to demonstrate that the development of diabetic cardiomyopathy is directly related to mitochondrial dysfunction and impaired calcium handling. The disease is characterised by hyperglycaemia, causing metabolic changes in diabetes, and it also appears to be associated with an increase in Drp1-dependent fission and cardiomyocyte hypertrophy. Drp1 is a key

regulator of mitochondrial fission, and some post-translational modifications, such as phosphorylation, can modulate its activity. In ZDF rats, high glucose (HG) increased protein levels of Drp1, whereas there was a decrease in the protein expression levels of Opa1 and Mfn2, which are pro-fusion proteins. This was also observed in neonatal rat cardiomyocytes (NRCMs) treated with high glucose by analysing the mitochondria, which, due to the Drp1 increased activity, appeared fragmented and had lower mitochondrial membrane potential. Moreover, in vitro neonatal cardiomyocytes were increased in size, and had a higher expression of two important markers of cardiac hypertrophy, i.e. β-MHC and ANP, showing that hyperglycaemia causes hypertrophy in NRCMs.

Calcium handling is crucial for the activity of cardiomyocytes. The Ca^{2+} release-activated calcium channel protein 1 (Orai1) regulates the intracellular calcium uptake, and it was observed that high glucose induced the overload of Ca^{2+} by the upregulation of Orai1 expression, and consequently, this leads to cardiac hypertrophy, since the dysregulation of Ca^{2+} handling is associated with this disease. To understand the effect of Orai1 dysregulation on cardiomyocytes, with the use of channel inhibitor (BTP2) or by performing a knockdown with the Cas9/sgRNA technique, the expression of Orai1 was downregulated in NRCMs of the high glucose group. Additionally, BTP2 decreased mitochondrial fission and improved mitochondrial dysfunction. Moreover, Orai1-calcium handling seems to influence the activity of two proteins involved in the post-translational modifications of Drp1. One is called extracellular signal-regulated kinase (ERK), whereas the other one is a calcineurin subunit called calmodulin-binding catalytic subunit A (CnA). ERK phosphorylates Drp1 at S616, and CnA is responsible for the dephosphorylation of Drp1 S637. These modifications made by the two enzymes are crucial to activate Drp1-dependent mitochondrial fission, and since the activity of these proteins is regulated by calcium uptake, the Orai1 channel induces cardiac hypertrophy by regulating Drp1 phosphorylation. In ZDF rats, the protein levels of ERK, CnA, and Orai1 were increased due to hyperglycaemia. Then, ERK and CnA expression augmented in NRCMs with BTP2, so the activation of these proteins may be associated with Orai1-mediated Ca^{2+} entry. Additionally, neonatal cardiomyocytes were treated with a molecule that inhibited ERK, whose expression decreased, and consequently, phosphorylation of Drp1 at S616 decreased too. The dephosphorylation of Drp1 at S637 by a CnA inhibitor was inhibited. Altogether, these results show that “mitochondrial fission induced by phosphorylation of Drp1 at S616 or dephosphorylation at S637 is a critical event in HG-induced cardiomyocyte hypertrophy” (Wu et al., 2021).

In this study, it was observed that the effects of high glucose seem to be reversible when cardiomyocytes are treated with a Drp1 cell-permeable inhibitor, Mdivi-1. This molecule prevents fission by inhibiting phosphorylation at S616 while increasing the phosphorylation at S637. In NRCMs, the cardiomyocyte size and the protein levels of β-MHC and ANP were decreased after Mdivi-1 treatment, meaning that the inhibitor can alleviate high glucose-induced cardiac hypertrophy. Moreover, mitochondrial fission was reduced; the phosphorylation of Drp1 at S616 and the dephosphorylation of Drp1 at S637 seemed to be both inhibited by measuring their expression. So, these results showed that Mdivi-1 can prevent mitochondrial fission induced by high glucose. To investigate the therapeutic outcome of Mdivi-1, diabetic mice were injected with the molecule. Before the injection, they had higher protein levels of β-MHC and ANP were compared with non-diabetic mice. The expression of phosphorylated $Drp1^{S616}$ was decreased, and the phosphorylation of Drp1 at S637 had increased, similarly to in vitro results. Moreover, mitochondrial morphology was rescued in diabetic mice after Mdivi-2 injection in mice. Overall, this selective inhibitor of Drp1 seems to be effective against cardiac hypertrophy in mice; however, the process through which Mdivi-1 works in the diabetic cardiomyopathy condition remains unknown.

To sum up, ZDF rats showed all the characteristics of DCM, such as hyperglycaemia and cardiac hypertrophy. The disease seems to be connected to mitochondrial dysfunction and impaired Ca^{2+} handling. Protein levels were measured both in vivo and in vitro, showing an increase in Orai1, CnA, and ERK expression and Drp1 activity. Additionally, mitochondrial staining allowed visualisation of accumulated fragmented mitochondria after HG-induced hypertrophy. In this study, it was demonstrated for the first time that Orai1, responsible for Ca^{2+} entry into the cell, might contribute to high glucose-induced cardiac hypertrophy via regulating ERK and CnA activity. Indeed, EKR is responsible for the phosphorylation of Drp1 at S616, while CnA decreases the phosphorylation of Drp1 at S637, and these modifications promote the fission activity of Drp1. Targeting Orai1, ERK, or CnA seems to decrease Drp1 activity and alleviate the effects of cardiac hypertrophy induced by high glucose levels. Thus, the regulation of all these proteins could be a valuable strategy to ameliorate cardiac hypertrophy associated with diabetic cardiomyopathy (Wu et al., 2021).

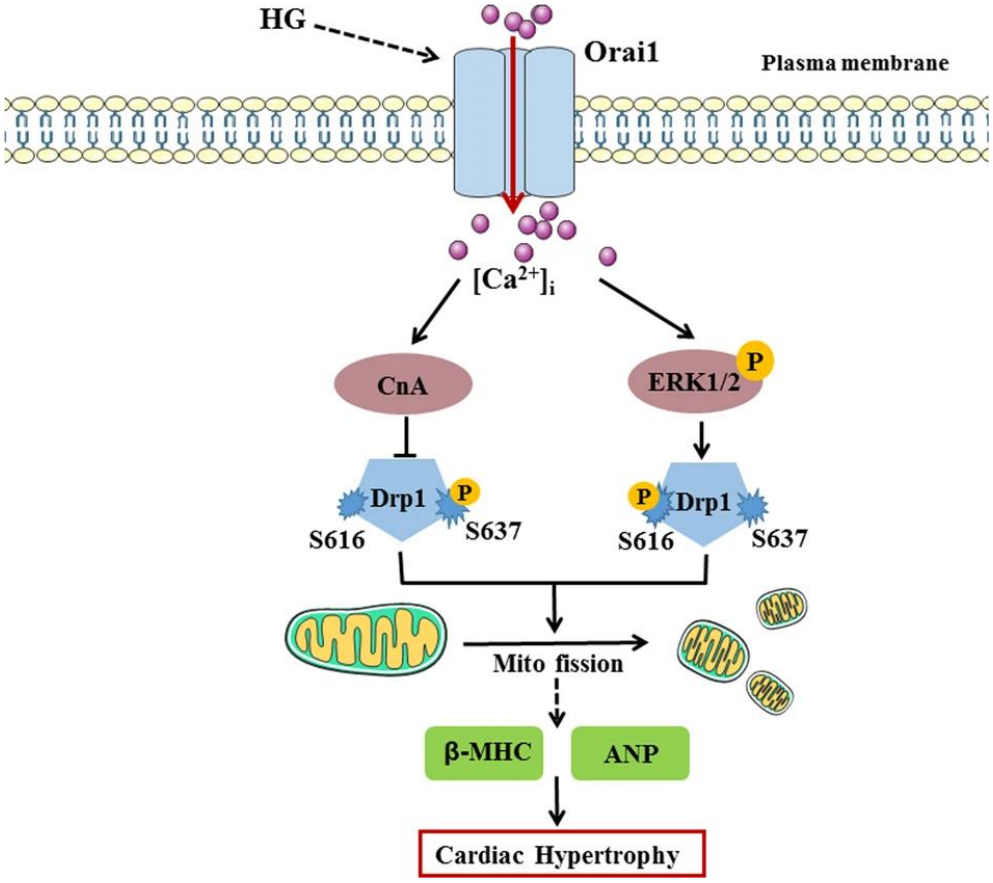

Figure 5. Schematic representation of high glucose (HG) induced mitochondrial fission that leads to cardiac hypertrophy and the Ca^{2+} overload-induced cellular pathways (Wu et al., 2021).

4.4 Atherosclerosis

Blood vessels are a crucial component of the cardiovascular system, as they provide oxygen, nutrients, and other essential substances to the entire body and the heart. The vascular endothelium regulates blood flow through vasoconstriction and vasodilation. Flow-mediated dilation (FMD) is correlated with the development of various diseases, including diabetes, obesity, and cerebrovascular and cardiovascular disorders, all of which are characterized by reduced laminar blood flow (Chehaitly et al., 2022). This reduction of FMD is mainly due to oxidative stress, which causes damage to the endothelial cells (ECs) of the vessels. Although these cells get their energy mostly from glycolysis, it seems that mitochondria are involved in the regulation of ECs' activity. Indeed, it was shown that the pro-fusion protein Opa1 is crucial in developmental and tumour angiogenesis. Additionally, during angiogenesis, this protein regulates the migration and branching of ECs and the formation of filopodia in tip ECs. In a

study by Chehaitly et al. (2022), the role of mitochondrial fusion is investigated both in vivo and in vitro to assess whether it could have a role in flow-mediated dilation, in the development of cardiovascular diseases and if it may provide a protective function of the vascular walls via regulation of the flow-mediated signalling. In the study, human umbilical vein endothelial cells were treated with siOPA1, a single-interference (si) RNA to silence the pro-fusion protein gene. These cells showed a reduction in migration and elongation after being exposed to laminar shear stress compared to the control group, indicating that the silencing of Opa1 prevents ECs from responding properly to FMD. The effects of flow shear stress were also investigated in vitro by using two models of transgenic mice with altered expression of Opa1. The first model was characterised by $Opa1^{+/-}$ male and female mice, whose mesenteric arteries were extracted and tested for FMD. A reduction in flow-mediated dilation was observed in these tissues, and the same outcome was detected in mesenteric arteries taken from mice ECs whose Opa1 gene was not expressed after its knockout. To prove that an impairment in FMD may cause oxidative stress and eventually atherosclerosis, wild type mice aortas produced normal levels of Opa1 and Drp1 when submitted to a laminar flow in vivo. On the other hand, a decreased expression of Opa1 and an increased level of Drp1 were detected in mice aortas when submitted to a disturbed flow. Another experiment to investigate the effects of Opa1 ablation in the vascular endothelium and its possible regulatory function during FMD consists of giving a high-fat diet, particularly the atherogenic diet (Western diet) to both Opa1-deficient mice and wild type mice to obstruct the blood vessels with lipid deposits and by doing that, generating the atherosclerotic mice. In both male and female mice lacking Opa1, there was a greater lipid deposit in their aortas compared to normal mice. The fat accumulation in the aorta was also investigated in $Opa1^{+/-}$ mice, and the results were like those of mice having Opa1 deletion in ECs. The lipid deposition was visualized by Oil red-O staining. These lipid deposits formed plaques in the endothelium of the aortas and impaired blood flow. Interestingly, no significant differences were detected for body weight, total cholesterol, triglycerides, and glycemia between normal mice and mice with Opa1 altered expression that were fed the same high-fat diet. An ex vivo analysis was done on the kidneys of Opa1 knockout mice against wild type to see how the ECs of the kidney's vessels could react to perfusion pressure. It was observed that in Opa1-deficient kidneys, the ATP production decreased, whereas the H_2O_2 levels were increased, indicating a higher level of oxidative stress in both male and female mice.

In summary, the study demonstrated that Opa1 may regulate the response to blood flow change during FDM in ECs of the vessels in both male and female mice. The

lack of this pro-fusion protein has been demonstrated to reduce FMD in vitro and in vivo. A lower level of expression was detected in ECs submitted to disturbed shear stress. Moreover, the deficiency seems to be connected to an increase in H_2O_2 production, which can be responsible for oxidative stress (Chehaitly et al., 2022).

4.5 Pulmonary arterial hypertension

Hypertension or high blood pressure is a disease characterized by impairments of the vascular tissue to function properly and increased vascular resistance that is related to higher blood pressure (Marsboom et al., 2012). When the increase in pressure causes constriction, obstruction, and inflammation of the pulmonary arteries, the condition is called pulmonary artery hypertension (PAH). This syndrome, if not treated, can lead to ventricular hypertrophy and, eventually, heart failure. The development of PAH is associated with augmented mitochondrial fission that is caused by an increased recruitment of the fission protein Drp1. In a study by Marsboom et al. (2012), the role of Drp1 was investigated both in rats and in humans. Pulmonary artery smooth muscle cells (PASMCs) from PAH patients were observed in vitro and compared with the control cells of the same tissue. The ones of PAH patients showed more fragmented mitochondria, consistent with the increased expression level of Drp1. High levels of Drp1 phosphorylated at Ser616 are detected, which is related to a high activity of the enzyme responsible for the phosphorylation, that is CDK1 (cyclin-dependent kinase 1). Indeed, CDK1 allows the activation of Drp1 in PAH, and its inhibition reduces Drp1 phosphorylation at Ser616. Additionally, the mitochondrial networks were decreased in PHA cells compared to the control group. When PASMCs of PAH patients were treated with a Drp1 inhibitor, i.e. Mdivi-1, the activity of the fission protein was decreased, and the cell-cycle progression was blocked. This is because the cell cycle is regulated by mitochondrial fission, which influences cell proliferation. Indeed, higher mitochondrial fragmentation is also correlated with an increased multiplication of PASMCs with PAH. By silencing Drp1 with a targeted siRNA, the cell proliferation was reduced, as with the Mdivi-1 treatment, demonstrating that the inhibitor molecule works by binding to the fission protein and, consequently, it reduces cell division. The observation of Drp1-PAH relation was also observed in human lung tissue recovered from autopsied PAH patients. The ex vivo arteries showed multiple lesions and were muscularized, both signs of hypertension. Similarly to the

PASMCs, the expression of both Drp1 and CDK1 was increased, meaning that the fission protein was activated.

It was discovered that an excessive proliferation of human PAH PASMCs is associated with an activation of HIF-1α, the hypoxia-inducible factor 1α. This protein regulates the cellular response to low levels of oxygen and is involved in the silencing of the superoxide dismutase-2 enzyme, which is responsible for eliminating reactive oxygen species (ROS). Additionally, it inhibits mitochondrial oxidative metabolism and increases the glycolysis process, which facilitates cell proliferation. Indeed, the study confirmed the activation of HIF-1α in PASMCs of PAH patients and an increased glycolysis was observed, indicating a correlation between the activation of this protein and the syndrome.

Moreover, it seems that the activation of hypoxia-inducible factor 1α may induce mitochondrial fragmentation in normal PASMCs, which eventually leads to the development of pulmonary arterial hypertension. This was tested by treating normal rat PASMCs with a HIF-1α activator, i.e. cobalt ($CoCl_2$). As predicted, cobalt activated HIF-1α, and, consequently, induced mitochondrial fragmentation and the phosphorylation of Drp1 at Ser616. Besides, the cobalt-induced fragmentation cannot occur when HIF-1α is silenced, and this demonstrates that "the fragmentation is attributable to HIF-1α activation" (Marsboom et al., 2012). In this model, Mdivi-1 could reverse the mitochondrial fission induced by HIF-1α, by rescuing mitochondrial morphology and restoring the organelle network. The same results were obtained by treating normal rat PASMCs with desferrioxamine, which also works as an HIF-1α activator and, eventually, stimulates mitochondrial fission. So, the cobalt-induced activation of HIF-1α seems to promote some features that are commonly associated with PAH. To verify if the cobalt-induced HIF-1α activation may cause PAH in vivo, wild type rats were treated with $CoCl_2$. Then, several factors related to PAH were evaluated, showing that the induction of HIF-1α activation caused pulmonary arterial hypertension in vivo. Indeed, several factors associated with the syndrome confirmed the hypothesis: the pulmonary artery acceleration time, a measure of vascular compliance, and the maximal walking distance both decreased in rats treated with cobalt. On the other hand, the activator increased pulmonary vascular resistance. Similarly to in vitro models of PAH, the Drp1 inhibitor Mdivi-1 was tested in vivo to see if it could attenuate the hypertension. After the injection, the maximal walking distance and the pulmonary artery acceleration time increased in cobalt-induced PAH rats. Whereas the pulmonary vascular resistance increased and the mitochondria appeared less fragmented after Mdivi-1 treatment. These results indicated that the Drp1 inhibitor could reverse the changes induced by

cobalt and attenuate the development of PAH. So, Mdivi-1 rescued PAH models in vivo and in vitro and could be used as a therapeutic method to treat pulmonary hypertension.

In summary, PAH seems to have a higher expression and activity of Drp1, which leads to more cell proliferation and increased mitochondrial fission. This is also associated with the activation of hypoxia-inducible factor 1α, which is detected in PAH models and is induced by cobalt or desferrioxamine in both in vivo and in vitro rat models. Therefore, there is a link between mitochondrial fission, HIF-1α, and the development of pulmonary arterial hypertension (Marsboom et al., 2012).

5. MITOCHONDRIAL DYNAMICS-CVDs CORRELATION: AN EXAMPLE IN HUMANS

Cardiovascular diseases are one of the main causes of death worldwide. These pathologies caused 32% of all global deaths in 2019, and out of the 17 million premature deaths (under the age of 70) due to noncommunicable diseases in 2019, 38% were caused by CVDs (World Health Organization, 2021). It is now well known that there is a correlation between these diseases and mitochondrial dynamics.

Thus, many research groups focus their studies on finding the root causes of these diseases, innovative techniques for diagnosis, and eventually, developing cures for treating the several kinds of cardiovascular disorders.

For example, a study by Hsiao et al. (2021) investigated the role of Mfn1 in patients affected by idiopathic dilated cardiomyopathy (IDCM). Twenty-two patients with IDCM underwent endomyocardial biopsy and were then given several medications to treat their condition. However, eight of them did not respond to the treatment, because an improvement of more than 10% on echocardiography was not shown during the evaluation of the left ventricular ejection fraction (LVEF), and so these patients were classified as non-responsive (Hsiao et al., 2021). To understand what could have caused the differentiation between responsive and non-responsive patients, the specimens from the biopsies were observed with a transmission electron microscope and in non-responsive patients, the mitochondria appeared smaller compared to those of the responsive patients. Since the mitochondrial changes in morphology are regulated by the mitochondrial dynamics, the protein and transcript expression of mitochondria-shaping proteins were investigated. Non-responders had a reduced expression of the pro-fusion protein Mfn1 and its transcript, while cardiomyocytes from

responsive individuals showed normal expression of Mfn1. Interestingly, in both responsive and non-responsive hearts, transcript expression of the other mitochondria-shaping proteins (*Mfn2*, *Opa1*, *Drp1* and *Fis1*) was very similar between the two groups. Thus, these results demonstrate that the cardiomyocytes of non-responsive patients are characterised by the impairment of mitochondrial fusion, resulting from the downregulation of Mfn1. To verify the role of Mfn1 in heart failure development, an in vitro model was generated by inducing the silencing of *Mfn1*, with a specific siRNA, in neonatal rat ventricular myocytes (NRVMs). A decrease in functional mitochondria was observed after Mfin1 silencing. The gene depletion reduced mitochondrial respiration and mitochondrial membrane potential, showing that an impairment in mitochondrial fusion is strictly associated with metabolic remodelling, particularly the suppression of mitochondrial respiration, which is crucial for ATP production. Indeed, it seems that as heart failure progresses, the ATP and phosphocreatine (i.e. a molecule that can enhance muscle activity by donating phosphate groups) levels decrease. So, Mfn1 ablation reduced cellular respiration, leading to lower energy production and, consequently, heart failure. The effects of Mfn1 suppression were also studied in a mouse model of Mfn1 knockout (KO) cardiomyocytes. Their hearts had a reduction in Mfn1 expression, whereas the other mitochondria-shaping proteins had normal expression, indicating that the knockout specifically and efficiently targeted Mfn1. The mitochondria of the KO cardiomyocytes appeared smaller under the electron microscope. Additionally, the mitochondrial metabolic activity was evaluated, and a reduction of the phosphocreatine/ATP ratio was observed, which is an indicator of a decrease in cellular respiration.

The KO mice were subjected to pressure overload model to assess the effect of Mfn1 ablation. The KO hearts had reduced systolic function and cardiac dilation during pressure overload. By observing the cardiomyocytes of KO mice with the electron microscope, it was revealed an accumulation of lysosomes and altered mitochondria. Moreover, KO hearts showed an increased fibrotic area compared to the wild type, and an increase in transcripts for fibrotic and inflammatory markers.

It has been shown that patients suffering from heart failure have high levels of circulating catecholamines (epinephrine and norepinephrine), which are associated with poor clinical outcomes. Indeed, the activation of the adrenergic signalling (β-adrenergic receptor (β-AR)/ cAMP/ PKA/ signalling pathway) promotes heart failure and cardiac hypertrophy. This well-studied pathway is a target for therapies that aim to inhibit the signalling, i.e. β-blockers as the first therapeutic option for failing hearts. To investigate whether this crucial pathway

affected the expression of Mfn1, NRVMs were treated with a β-AR agonist (isoproterenol), which decreased the expression of Mfn1. This reduction in Mfn1 expression was inhibited by treating cells with a PKA inhibitor.

The adrenergic signalling controls the expression of Mfn1 via regulating the modifications that this fusion protein undergoes after its transcription. MicroRNAs are also involved in post-transcriptional modifications of Mfn1 mRNA; in particular, miR-140-5p was characterized as the miRNA that inhibits Mfn1. Indeed, the gene expression of the fusion protein was inhibited when the expression of miR-140-5p was increased after administration of cAMP. Moreover, the expression of this miRNA was higher in cardiomyocytes of non-responsive patients compared to the level observed in responsive individuals. So, the adrenergic pathway can decrease the activity of Mfn1 in vitro through the activation of miR-140-5p, which can suppress Mfn1 expression. Since the downregulation of Mfn1 is associated with IDCM in non-response patients, and they show elevated circulating levels of catecholamines, the suppression of the β-AR/ cAMP/ PKA/miR-140-5p signalling pathway can be a therapeutic target for heart failure (Hsiao et al., 2021).

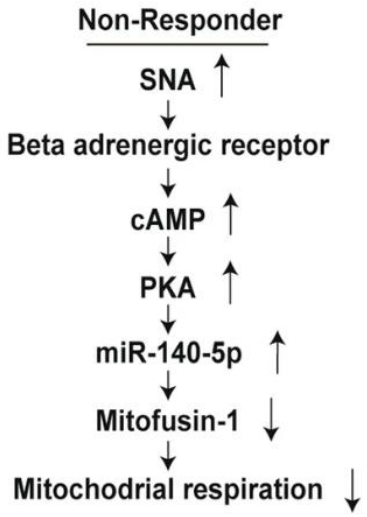

Figure 7. The activation of the adrenergic pathway leads to the downregulation of Mfn1 and is associated with heart failure in non-responsive IDCM patients (Hsiao et al., 2021, modified)

6. CONCLUSIONS

Mitochondria are double membrane-bound organelles responsible for converting cells' chemical energy in the form of ATP and for this reason, they are referred to as the powerhouses of the cell. This metabolic activity is essential for the cell survival and is deeply connected to the organelle's morphology. Mitochondrial dynamics regulate the shape of mitochondria, and they can be defined the balance between two opposing processes: fusion, which allows the generation of one bigger organelle by merging smaller mitochondria, and fission, a process of mitochondrial division (Yu et al., 2020). These processes are regulated by different proteins, called mitochondria-shaping proteins. Hence, mitochondrial dynamics guarantee mitochondrial metabolic activity through the coordinated action of these proteins.

Understandably, mitochondrial dysfunction is a direct consequence of impaired mitochondrial dynamics, and this is especially detrimental for the heart and the brain, since they are highly energy-demanding organs. Indeed, mitochondrial dynamics have been implicated the development of various pathologies, including neurodegenerative syndromes, cardiovascular diseases, and even cancer.

Numerous studies have investigated the correlation between mitochondria-shaping proteins and cardiovascular diseases to provide a deeper understanding of the molecular causes behind various cardiovascular pathologies and, ultimately, discover therapeutic methodologies that target mitochondrial dynamics.

For example, in acute myocardial infarction (IRI), downregulation of Mfn1 and Mfn2 has been shown to reduce infarct size in Mfn1/Mfn2 DKO mice. Inhibiting these proteins has a protective effect in IRI; however prolonged downregulation of Mfn1 and Mfn2 significantly impair cellular respiration and mitochondrial fusion, ultimately causing severe myocardial damage. Therefore transient suppression of Mfn1 and Mfn2 may offer a cardioprotection in IRI (Hall et al., 2016).

In another study, the ablation of YME1L leads to overactivation of OMA1, a protease that increases the Opa1 processing augmenting s-Opa1. The accumulation of Opa1 short form is associated with mitochondrial fragmentation and a metabolic change from fatty acid consumption to glucose consumption, promoting cardiac dilation and heart failure in *Yme1l*-KO mouse models (Wai et al., 2015). On the other hand, a reduction of Opa1 expression seems to be associated with endothelial cells' dysfunction in atherosclerosis. Indeed, the ablation of Opa1 both in vitro and in vivo reduces the responsiveness of cells to flow-mediated dilation of blood vessels (Chehaitly et al., 2022).

Human studies also highlighted the relevance of mitochondria-shaping proteins in the development of CDVs. For instance, Mfn1 downregulation is associated with the development of idiopathic dilated cardiomyopathy in patients who do not respond to cardiomyopathy treatment. Mfn1 reduced activity is promoted by adrenergic signalling, activated in cardiomyopathy; therefore, this pathway can be a therapeutic target to rescue mitochondrial morphology in failing hearts (Hsiao et al., 2021)

All these studies emphasise the importance of understanding the molecular mechanisms underlying the action of mitochondria-shaping proteins and their role in cardiovascular diseases. Moreover, targeting mitochondrial dynamics presents a promising therapeutic avenue in CVDs. For example, the Drp1 inhibitor Mdivi-1 can restore mitochondrial morphology by downregulating Drp1, whose overexpression promotes mitochondrial fragmentation and induces dilated cardiomyopathy in male diabetic (ZDF) rats (Wu et al., 2021). Additionally, Mdivi-1 has potential as a treatment for pulmonary artery hypertension, acting through the inhibition of HIF-1α-Drp1 pathway in both in vitro and mouse models (Marsboom et al., 2012).

7. BIBLIOGRAPHY

[1]Chehaitly, A., Guihot, A.-L., Proux, C., Grimaud, L., Aurrière, J., Legouriellec, B., Rivron, J., Vessieres, E., Tétaud, C., Zorzano, A., Procaccio, V., Joubaud, F., Reynier, P., Lenaers, G., Loufrani, L., & Henrion, D. (2022). Altered Mitochondrial Opa1-Related Fusion in Mouse Promotes Endothelial Cell Dysfunction and Atherosclerosis. *Antioxidants*, *11*(6). https://doi.org/10.3390/antiox11061078

[2]Hall, A. R., Burke, N., Dongworth, R. K., Kalkhoran, S. B., Dyson, A., Vicencio, J. M., Dorn, G. W., Yellon, D. M., & Hausenloy, D. J. (2016). Hearts deficient in both Mfn1 and Mfn2 are protected against acute myocardial infarction. *Cell Death & Disease*, *7*(5), e2238–e2238. https://doi.org/10.1038/cddis.2016.139

[3]Hsiao, Y. T., Shimizu, I., Wakasugi, T., Yoshida, Y., Ikegami, R., Hayashi, Y., Suda, M., Katsuumi, G., Nakao, M., Ozawa, T., Izumi, D., Kashimura, T., Ozaki, K., Soga, T., & Minamino, T. (2021). Cardiac mitofusin-1 is reduced in non-responding patients with idiopathic dilated cardiomyopathy. *Scientific Reports*, *11*(1), 6722. https://doi.org/10.1038/s41598-021-86209-y

[4]Marsboom, G., Toth, P. T., Ryan, J. J., Hong, Z., Wu, X., Fang, Y.-H., Thenappan, T., Piao, L., Zhang, H. J., Pogoriler, J., Chen, Y., Morrow, E., Weir, E. K., Rehman, J., & Archer, S. L. (2012). Dynamin-Related Protein 1–Mediated Mitochondrial Mitotic Fission Permits Hyperproliferation of Vascular Smooth Muscle Cells and Offers a Novel Therapeutic Target in Pulmonary Hypertension. *Circulation Research*, *110*(11), 1484–1497. https://doi.org/10.1161/CIRCRESAHA.111.263848

[5]Quintana-Cabrera, R., & Scorrano, L. (2023). Determinants and outcomes of mitochondrial dynamics. *Molecular Cell*, *83*(6), 857–876. https://doi.org/10.1016/j.molcel.2023.02.012

[6]Wai, T., García-Prieto, J., Baker, M. J., Merkwirth, C., Benit, P., Rustin, P., Rupérez, F. J., Barbas, C., Ibañez, B., & Langer, T. (2015). Imbalanced OPA1 processing and mitochondrial fragmentation cause heart failure in

mice. *Science*, *350*(6265), aad0116. https://doi.org/10.1126/science.aad0116

[7]Wu, Q.-R., Zheng, D.-L., Liu, P.-M., Yang, H., Li, L.-A., Kuang, S.-J., Lai, Y.-Y., Rao, F., Xue, Y.-M., Lin, J.-J., Liu, S.-X., Chen, C.-B., & Deng, C.-Y. (2021). High glucose induces Drp1-mediated mitochondrial fission via the Orai1 calcium channel to participate in diabetic cardiomyocyte hypertrophy. *Cell Death & Disease*, *12*(2), 216. https://doi.org/10.1038/s41419-021-03502-4

[8]Yu, R., Lendahl, U., Nistér, M., & Zhao, J. (2020). Regulation of Mammalian Mitochondrial Dynamics: Opportunities and Challenges. *Frontiers in Endocrinology*, *Volume 11-2020*. https://doi.org/10.3389/fendo.2020.00374

[9]World Health Organization (2021). Cardiovascular diseases (CVDs). https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)

8. Riassunto esteso

I mitocondri sono degli organelli che caratterizzano la maggior parte degli eucarioti e sono essenziali per la sopravvivenza della cellula. Infatti, svolgono diverse funzioni, tra le quali la produzione di energia chimica attraverso la respirazione cellulare, regolano l'omeostasi del calcio, permettono la detossificazione dai ROS, e sono convolti in diversi aspetti della vita cellulare come l'apoptosi, la proliferazione e la differenziazione cellulare (Yu et al., 2020). Per garantire queste funzioni, la morfologia mitocondriale costituisce un importante fattore per quanto riguarda la sua attività; perciò, i meccanismi che regolano la struttura dei mitocondri sono essenziali per assicurare le sue funzioni. La fusione mitocondriale permette l'unione di uno o più mitocondri per recuperare quelli danneggiati oppure rispondere ad una richiesta energetica più elevata. Al contrario, la fissione mitocondriale controlla la divisione dei mitocondri e questa agisce soprattutto durante la ridistribuzione di questi organelli o del loro materiale genetico tra cellule vecchie e nuove, oppure a cellule figlie. Questi due processi regolano la morfologia mitocondriale e l'equilibrio tra fusione e fissione è definito come dinamiche mitocondriali (Quintana-Cabrera & Scorrano, 2023). Nei mammiferi, la fusione avviene grazie all'attività di tre proteine chiave che sono Mfn1 e Mfn2, responsabili per l'unione tra due membrane mitocondriali esterne, mentre la proteina Opa1 controlla la fusione delle membrane mitocondriali interne. Per quanto riguarda la fissione, i mitocondri dei mammiferi vengono frammentati grazie alla proteina Drp1, la quale può essere reclutata sulla superficie mitocondriale da diverse proteine adattatrici tra cui Fis1, Mff, MiD49 e MiD51 (Yu et al., 2020; Quintana-Cabrera & Scorrano, 2023).

È ormai noto che esiste una correlazione tra le dinamiche mitocondriali e lo sviluppo di alcune patologie umane, tra cui le malattie cardiovascolari. Diversi studi hanno dimostrato il ruolo delle dinamiche mitocondriali in queste patologie che colpiscono il sistema cardiocircolatorio. Ad esempio, è stato dimostrato che se le proteine Mfn1 e Mfn2 coinvolte nel processo di fusione mitocondriale, vengono soppresse, questo può alleviare gli effetti negativi provocati dal danno da riperfusione dopo infarto cardiaco (Hall et al., 2016). Per quanto riguarda la fissione mitocondriale, l'aumento di Drp1 nei mitocondri dei cardiomiociti sembra favorire l'ipertrofia cardiaca, associata alla cardiomiopatia diabetica (Wu et al., 2021). Inoltre, Drp1 stimola la frammentazione mitocondriale e la proliferazione cellulare, favorendo l'insorgenza dell'ipertensione arteriale polmonare (Marsboom et al., 2012). Per quanto riguarda Opa1, il suo

processamento può aumentare la fissione mitocondriale e compromettere attività metabolica dei cardiomiociti, promuovendo lo scompenso cardiaco (Wai et al., 2015). D'altra parte, la ridotta espressione di Opa1 può essere associata allo sviluppo dell'aterosclerosi (Chehaitly et al., 2022). In conclusione, lo studio delle dinamiche mitocondriali permette di comprendere i meccanismi molecolari che portano allo sviluppo delle malattie cardiovascolari e realizzare delle terapie più mirate.