UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Universitat Politècnica de València

Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural

Biotechnology degree

4th course

Mitochondrial Dynamic Processes in Left Ventricular Tissue from Heart Failure Patients

José Álvaro Ballesteros González

UPV tutor:
Dra. María Adelaida García Gimeno

Directors:
Dra. Esther Roselló Lletí
Dr. Manuel Portolés Sanz

Valencia, September 2018

Memory

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ABSTRACT

Cardiac diseases are globally the first cause of dead. Heart failure (HF) comprise several subtypes of cardiomyopathies caused by different physiological and molecular defects. Ischemic cardiomyopathy (ICM) is the main HF etiology and is related with a progressive decrease of heart pump activity, resulting in a suboptimal oxygen supply. Mitochondria play a crucial role in cardiomyocytes activity because of their implication in heart contractility and oxygen and ATP production. Recent studies have found a relation between alterations in mitochondrial dynamics and HF predisposition. Fission, fusion, mitophagy and cristae formation are important in mitochondrial dynamics. These processes determine vital properties in cardiac tissue integrity and activity. However, molecules involved in these mechanisms have not been extensively studied in HF.

In this study, mitochondrial dynamics molecules are evaluated in left ventricular tissue from explanted human hearts. Transcriptomic (RNA-seq) and statistical analysis are performed to know which mRNAs are up-regulated or down-regulated in ICM patients compared with a control group.

22 mitochondria genes were detected as altered in fission, mitophagy and cristae formation processes. None fusion-related genes were detected as altered, while *MTFR1L* (FC= 1.38; $p<0.05$) and *RAB7L1* (FC= -1.43; $p<0.05$) are altered in fission. In mitophagy, mitochondrial genes are all downregulated, such as *PARK2* (FC= -1.49; $p<0.05$) or CHDH (FC: -2.15; $p<0.05$) and the Bcl-2 family proteins, such as *BCL2L1* (FC= -1.51; $p<0.05$), *BCL2L13* (FC= -1.38; $p<0.01$) and *BCL2L2* (FC= -1.73; $p<0.01$); some non-mitochondrial genes, such as *CALCOCO2* (FC= -1.62; $p<0.0001$), are downregulated, while others are upregulated, such as *MAP1LC3A* (FC= 1.67; $p<0.05$) and *TBC1D17* (FC= 1.50; $p<0.05$). Finally, in cristae formation, all genes that form ATPase and MICOS complex are upregulated, such *ATP5D* (FC= 1.62; $p<0.05$), *ATP5I* (FC= 2.04; $p<0.01$) or *APOO* (FC= 1.65; $p<0.01$). Transmission electron microscopy micrographs are obtained to complement these results, showing an alteration in mitochondrial structure in ischemic tissue.

In conclusion, our findings indicate that mitophagy and cristae formation are the dynamics processes with the most relevant changes in ICM hearts. Thus, these processes could increase damage of the cardiac mitochondria in ICM, interfering critical activities of the cardiomyocyte, such as alterations in the ATP production that is necessary for a correct cardiac ventricular function.

KEYWORDS

Mitochondria, fission, fusion, mitophagy, cristae formation, ischemic cardiomyopathy (ICM), heart failure (HF).

Procesos dinámicos mitocondriales en tejido ventricular izquierdo de pacientes con insuficiencia cardíaca

José Álvaro Ballesteros González

Dra. Esther Roselló Lletí

Dr. Manuel Portolés Sanz

Dra. María Adelaida García Gimeno

RESUMEN

Valencia, septiembre de 2018

Las enfermedades cardíacas representan la principal causa de muerte en todo el mundo. La insuficiencia cardíaca (IC) comprende varios subtipos de cardiomiopatías causadas por diferentes defectos fisiológicos y moleculares. Entre ellas, la miocardiopatía isquémica (MCI) es la principal etiología de IC, y está relacionada con un descenso progresivo en el bombeo cardíaco, lo que genera un suministro menor de oxígeno en el resto del organismo. Las mitocondrias juegan un papel crucial en la actividad de los cardiomiocitos por su implicación en la contractilidad cardíaca y la producción de oxígeno y ATP. Estudios recientes han relacionado alteraciones en diferentes mecanismos mitocondriales con la predisposición a sufrir IC. Los procesos mitocondriales de fusión, fisión, mitofagia y formación de crestas son determinantes en la dinámica mitocondrial y son vitales en la integridad y la actividad del tejido cardíaco. A pesar de ello, las moléculas implicadas en estos procesos todavía no han sido estudiadas en profundidad en IC.

En este trabajo, se han evaluado diferentes moléculas implicadas en la dinámica mitocondrial en tejido cardíaco del ventrículo izquierdo de corazones humanos explantados. A partir de un análisis transcriptómico (RNA-seq) y estadístico, se han identificado una serie de mRNAs que cambian su expresión en pacientes con MCI, en comparación con un grupo control. 22 genes mitocondriales fueron detectados como alterados en los procesos de fisión, mitofagia y formación de crestas. Ningún gen de fusión modifica su expresión en pacientes MCI, mientras que los genes de fisión *MTFR1L* (FC= 1.38; $p<0.05$) y *RAB7L1* (FC= -1.43; $p<0.05$) sí lo estaban. En mitofagia, los genes que se encuentran en la mitocondria están todos infraexpresados, como los genes *PARK2* (FC= -1.49), CHDH (FC= -2.15; $p<0.05$) y la familia génica Bcl-2, como *BCL2L1* (FC= -1.51; $p<0.05$), *BCL2L13* (FC= -1.38; $p<0.01$) y *BCL2L2* (FC= -1.73; $p<0.01$); algunos de los genes no mitocondriales estaban infraexpresados, como *CALCOCO2* (FC= -1.62; $p<0.0001$), mientras que otros estaban sobreexpresados, como *MAP1LC3A* (FC= 1.67; $p<0.05$) y *TBC1D17* (FC= 1.50; $p<0.05$). En la formación de crestas, todos los genes que forman los complejos ATPasa y MICOS estaban sobreexpresados, como *ATP5D* (FC= 1.62; $p<0.05$), *ATP5I* (FC= 2.04; $p<0.01$) o *APOO* (FC= 1.65; $p<0.01$). Se obtuvieron micrografías a través de microscopía electrónica de transmisión (MET) para observar los efectos de la IC en las estructuras mitocondriales.

En conclusión, este trabajo muestra que la mitofagia y la formación de crestas son las dinámicas mitocondriales con cambios más relevantes en corazones IC. Por ello, estos procesos podrían incrementar el daño en mitocondrias cardíacas in IC, interfiriendo en las principales actividades del cardiomiocito, como en la producción de ATP, necesaria para una correcta función ventricular cardíaca.

PALABRAS CLAVE

Mitocondria, fisión, fusión, mitofagia, formación de crestas, miocardiopatía isquémica (MCI), insuficiencia cardíaca.

ACKNOWLEDGMENTS

To my family and friends.

To the teachers that help me to make science my passion.

To the Myocardial Dysfunction and Cardiac Transplantation Unit from Health Research Institute La Fe, for let me develop this incredible project and encouraged me to make an effort on it.

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1. INTRODUCTION

1.1. Cardiovascular diseases

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, above other important diseases such as cancer or respiratory infections. According with the World Health Organization (WHO), 17.7 million people die each year from CVDs, what represents an estimated 31% of all deaths (GBD 2015 Mortality and Causes of Death Collaborators., 2016). Deaths occur mainly in low and middle-income countries, so CVDs are a critical issue that affects public health in our current society. In Spain, CVDs are also the first cause of death and disability, as in many other regions in Europe (Bertuccio et al., 2011), representing a 29,66% of the total deaths in our country, according to the National Institute of Statistics (INE) (INE, 2016).

CVDs are highly related with several behavioural risk factors, such as physical inactivity, unhealthy diet or abuse of alcohol, but also with other uncontrollable risk factors, such as age, inherited genetic disposition or physiological factors (Sun et al., 2015). The development of efficient treatments for CVDs is an important goal for cardiac researchers. Early detection techniques are also mandatory to carry out effective CVDs alternative treatments (Celermajer et al., 2012).

1.2. Heart failure

CVDs include several subtypes that involve affections in heart or blood vessels. Heart failure (HF) and cardiomyopathies are mains subtypes of CVDs, affecting about 40 million people (GBD 2015 Mortality and Causes of Death Collaborators., 2016), a 2% of adults and a 6-10% of the people with more than 65 years old (Metra & Teerlink, 2017). Thus, HF represent one of the main groups of CVDs pathologies.

HF is characterized by a progressive or spontaneous decrease on heart pump activity, leading to a lower oxygen supply of the heart and different organs. Principal symptoms of HF are breath difficulties, chronic fatigue and peripheral edema, in addition to different organs failures related with the lack of blood supply. Many physiological reasons may be involved in HF, such as coronary artery disease, myocardial infarction, high blood pressure, heart valvular disfunction, atrial fibrillation, or infection of cardiomyocytes (NATIONAL CLINICAL GUIDELINE CENTRE (UK), 2010).

Latest improvements in cardiac transplantation and restoring adequate blood flow techniques have raised survival rates to higher percentage in the last years. However, premature prediction of the predisposition to suffer these cardiac diseases could improve survival rates. That's why several researches have been focused on the development of methodologies for the assessment of HF (Tripoliti et al., 2016).

1.3. Ischemic cardiomyopathy

Ischemic cardiomyopathy (ICM) is the main HF etiology, being the cause of more than 60% of all HF cases. ICM is caused by an alteration of the coronary arteries blood flow due to a narrowing of the vessel lumen (Briceno et al., 2016). An acute myocardial infarction is typical in ICM patients; however, some ICM patients do not present infarction despite suffering coronary artery disease. Atherosclerosis, diabetes or inflammation of arteries are the predominant causes of ICM, affecting the narrowing of the coronary arteries (Felker et al., 2002)..

Ischemia means local restriction of blood supply due to mechanical obstruction of blood vessel lumen. A poor blood flow produces deficient oxygen supply of cardiac muscular layer cells, causing cell death and tissue damage. A complete recovery of cardiomyocytes normal activity after

an ischemic event is almost impossible and several molecular signals remain in cells (Chiong et al., 2011).

Basic research about molecular ICM causes and consequences could help to know how different molecules are involved in processes that are implicated in this disease, endowing scientific community innovative approaches to develop new diagnosis and tracking tools in cardiac personalized medicine.

1.4. Mitochondria as cells "power houses"

Mitochondria, found in almost every eukaryotic cell, contain distinct parts organized by a double membrane system: the outer mitochondrial membrane (OMM) protects mitochondria from external stresses; the intermembrane space, the space between both membranes, is where the oxidative phosphorylation is undertaken; the inner mitochondria membrane (IMM) is where oxidative phosphorylation and ATP synthase complexes are embedded; the cristae space, formed by the invaginations of the IMM, that increases IMM surface and, consequently, energy production; and the mitochondrial matrix, the space enclosed by the IMM membranes, with several functions in ATP production.

Mitochondria are the major site of ATP obtention in a cell. ATP is used as principal chemical energy molecule. Many proteins that are embedded in the IMM are involved in the oxidation of the products obtained from glucose to ATP. The electron transport chain (ETC) allows oxidative phosphorylation of NADH and $FADH_2$ molecules, generating a strong electrochemical gradient across the IMM. Protons obtained from NADH and $FADH_2$ can return to matrix through the ATP synthase complex, allowing the synthesis of ATP from ADP and inorganic phosphate. In order to expand the surface area of the IMM, a high compartmentalization is undergone forming numerous sections, known as mitochondrial cristae, enhancing the ability to produce ATP by each mitochondria (Zick et al., 2009).

1.5. Mitochondria in cardiac diseases

The molecular mechanisms of HF diseases remain unclear due to the complexity of the machineries that control several aspects of cardiac cells, such as contractility or depolarization/repolarization cycle. Different researchers have identified molecular changes in HF patients that are directly related with cardiomyopathies, such as calcium homeostasis alterations (Luo & Anderson, 2013), changes in size and complexity of Golgi apparatus (Tarazón et al., 2017) or proteins involved in energy metabolism and substrate utilization (Roselló-Lletí et al., 2014) (Roselló-Lletí et al., 2015).

Nevertheless, mitochondrial activity has been demonstrated to be highly related with HF, being a vital factor in the development of different cardiomyopathies (Bayeva et al., 2013). In cardiomyocytes, energy produced by mitochondria is mainly used in heart contractility, so alterations in mitochondria may affect heart contractility, decreasing cardiac activity (Doenst et al., 2013). As shown in some studies, several proteins altered in ICM and DCM patients are mitochondrial molecules (Roselló-Lletí et al., 2012). This proteomic study of human LV tissue demonstrated that half of the altered proteins obtained in this study were related with mitochondrial activity in ICM patients, as *ATP5O*, *ATP5A* or *PHB*. Although several studies have been undertaken relating mitochondria and HF etiologies, a whole study of the molecules involved in mitochondrial dynamics have not been carried out yet.

1.6. Mitochondrial dynamics

Mitochondria are high dynamic organelles that are in continuous cycles of fission, fusion and mitophagy. These mechanisms allow cells to have an accurate quality control system of their mitochondrial units (Suárez-Rivero et al., 2016). Cristae formation is also related with mitochondrial ability to produce enough quantities of ATP to maintain appropriate heart contractility (Zick et al., 2009). So, these dynamics processes, fission, fusion, mitophagy and cristae formation, are supposed to be high related with mitochondrial function, activity, integrity and energy production.

Disorders in mitochondrial dynamics have been demonstrated to be related with some kinds of diseases, such as diabetes or Hungtinton's disease (Gorman et al., 2016). Also, they are related with the predisposition to suffer other ones, such as different cardiomyopathies, for example ICM (Brunel-Guitton et al., 2015). Understanding how mitochondrial dynamics affects to cardiomyocytes activity could help scientific community to clarify which role mitochondria play in the pathophysiology of HF. A molecular approach about how these processes are carried out is mandatory to have an accurate point of view of these mechanisms.

1.6.1. Fusion and fission in mitochondria

Mitochondrial fusion and fission events are continuously occurring in somatic cells as part of their quality control system. Their main functions are to determine shape, size and distribution of mitochondria (Anand et al., 2014). They undergo according to cellular environment changes, adapting mitochondria activities to cellular necessities. Although they occur in a coordinated manner, each process is oriented to perform different tasks. Fusion contributes to mitochondrial maintenance, while fission main objective is to create new mitochondrial units, but also to segregate dysfunctional and damaged ones (van der Bliek et al., 2013). Both processes are controlled by accurate systems where multiple proteins are involved, that control these dynamics processes (Chan, 2012).

In mammals, two mitofusins proteins are implicated in mitochondrial fusion, Mitofusin-1 (*MFN1*) and Mitofusin-2 (*MFN2*) (Santel et al., 2003). These proteins are embedded in the OMM and regulate fusion between external membranes of mitochondria by GTPase activity (Ishihara et al., 2004). Dynamin-like 120 kDa protein, encoded by *OPA1* gene, is involved in the regulation of fusion between two mitochondria IMM, cooperating with mitofusin proteins by GTPase activity (Z. Song et al., 2007). Metalloendopeptidase *OMA1* and ATP-dependent zinc metalloprotease *YME1L* are also involved in fusion process due to their implication in *OPA1* cleavage in functional units (Anand et al., 2014).

Fission process is regulated by Dynamin-1-like protein, encoded by *DNM1L* gene, that mediates membrane fission through oligomerization into membrane-associated tubular structures and GTP hydrolysis-dependent mechanism (Smirnova et al., 2001). Additional membrane-associated adaptor proteins, such as Mitochondrial fission 1 protein (*FIS1*), Mitochondrial dynamics protein MID51 (*MIEF1*), Mitochondrial dynamics protein MID49 (*MIEF2),* Mitochondrial fission factor (*MFF)* or the Rab32 protein family (such as Ras-related protein Rab-7L1 [*RAB7L1])*, are needed for *DNM1L* protein recruitment and it integration into OMM (Koirala et al., 2013).

1.6.2. Mitophagy

Mitochondria suffer degradation because they are highly exposed to intra- and extra-events that affect their viability and normal activity, such as degradation by reactive oxygen species (ROS) (Melser et al., 2015). As mitochondria accomplish critical function in energy supply in cells, a selective replacement of useless mitochondria is mandatory for keeping cell health.

Mitophagy, or autophagy of mitochondria, is the process whereby cells recycle several biomolecules that are part of damaged or stressed mitochondria (Lemasters, 2005). It is an accurate process where autophagosomes and lysosomes participate degrading injured mitochondrial units. *PINK1/PRKN* pathway is the main and best characterized mitophagy pathway, but other signaling pathways promotes mitophagy in a cell. In damaged and depolarized mitochondria, serine/threonine-protein kinase *PINK1* protein is not able to translocate from OMM to IMM and remains in the intermembrane space where phosphorylates E3 ubiquitin-protein ligase parkin (*PRKN*) protein. Then, *PRKN* ubiquitylates many OMM proteins, that regulate selection of damaged mitochondria by autophagosomes (Jin & Youle, 2012). Several molecules are implicated in this pathway, as Calcium-binding and coiled-coil domain-containing protein 2 (*CALCOCO2)* (Lazarou et al., 2015).

Other molecules are involved in this process, such as different proteins that form the translocase system (Mitochondrial import receptor subunit *TOM20* homolog [*TOMM20];* Mitochondrial import receptor subunit *TOM40* homolog *[TOMM40]*) or that actuate as mitophagy receptors in the OMM surface, such as *BCL2*/adenovirus E1B 19 kDa protein-interacting protein 3-like *(BNIP3/NIX)* (Ney, 2015) or *FUN14* domain-containing protein 1 (*FUNDC2)* (Liu et al., 2012).

1.6.3. Cristae formation

Energy production is a central issue that mitochondria carry out through the ATP transformation from ADP and Pi. This process is accomplished by different complexes that form the electron transport chain (ETC), included several cytochromes and ATP synthase, embedded in the IMM. However, a shallow surface of this membrane would not be enough to contain all complexes needed to produce sufficient energy in a cell. For that reason, there are several compartments of this membrane called cristae, increasing IMM surface and ETC complexes units attached (Griparic & Bliek, 2001). Cristae membrane and inner boundary membrane are connected by crista junctions via tubular structures (Rabl et al., 2009).

Two multiprotein complexes are involved directly with cristae formation: MICOS and ATP synthase complexes. MICOS complex is formed by several mitofilin proteins that are required for keeping cristae membranes connected to the inner boundary membrane (Malsburg et al., 2011). This complex is required to endow cristae enough stability to integrate ETC complexes. One of these ETC complexes is ATP synthase complex or Complex V, that is embedded in crista membranes and produces ATP from ADP and Pi (Okuno et al., 2011). Also, this complex is required for generating mitochondrial cristae because the oligomerization of ATP synthase complex determine curvature and shape of crista membrane (Zick et al., 2009). Some ATP synthase subunits have been demonstrated to play critical role in the development of ICM (Roselló-Lletí et al., 2015).

As in previous processes, several proteins and molecules are also involved in cristae formation, such as the prohibitin family (Prohibitin [*PHB*]; Prohibitin-2 [*PHB2]*) (Roselló-Lletí et al., 2012) or proteins implicated in the metabolism and interaction with cardiolipin lipids, as Tafazzin

(*TAZ*) (Acehan et al., 2007) and Mitochondrial proton/calcium exchanger protein (*LETM1*) (Tamai et al., 2008).

2. OBJECTIVES

The main objective of this work is to evaluate the molecules involved in the mitochondrial dynamic processes (fusion, fission, mitophagy and cristae formation) in left ventricular (LV) tissue samples from explanted human hearts. An extensive bibliography research is assessed to identify mitochondrial proteins implicated in these dynamics processes. A whole transcriptomic assay (RNA-seq analysis) is performed to compare mRNA expression data from ICM hearts with a control group. Transmission electron microscopy (TEM) analysis is undertaken to observe potential mitochondrial alterations in cardiomyocytes from ICM.

3. MATERIALS AND METHODS

This study was carried out according to protocols from Myocardial Dysfunction and Cardiac Transplantation Unit from Health Research Institute La Fe.

3.1. Sample collection

LV tissue samples from explanted human hearts were obtained for RNA-seq analysis. A total of 23 samples formed our pull of study: 13 from patients with ICM and 10 from a non-diseased control group (CNT). Explanted hearts were available in all cases, reducing time between reception and storage of samples. Access to operating rooms during interventions was allowed. A coordinated process for samples acquisition permits the obtention of high-quality samples, as evidenced by the RNA integrity numbers (RIN) (≥9). Tissue samples were collected from a region near the apex of the left ventricle. 5 cm^2 size samples were extracted from each explanted heart. After the extraction, each sample was kept in a 0.9% NaCl solution, and conserved at 4 °C for a maximum of 6 h after the extraction because the lack of coronary blood circulation. Then, samples were stored at−80 °C until next assay (Tarazón et al., 2017).

Various aspects were requested to obtain as much clinical information as possible from each patient. Clinical history, electrocardiograms, Doppler echocardiography, hemodynamic studies, and coronary angiography data were available to classify patients according to the New York Heart Association functional criteria. Patients received medical treatment according to the guidelines of the European Society of Cardiology (McMurray et al., 2012) Subjects with primary valvular disease were excluded from the study. Table 1 represents clinical characteristics of ICM patients.

Control samples were acquired from non-diseased donor hearts that had been discarded for cardiac transplantation because of size or blood type incompatibility. Causes of death for these donors were either cerebrovascular events or motor vehicle accidents, with no cardiac damage in any case. All controls showed normal LV function [ejection fraction (EF) > 50%], as determined by Doppler echocardiography, and no history of cardiac disease. Data from control subjects was no available for the study, in accordance with the Spanish Organic Law on Data Protection 15/1999.

The present study was accepted by the Ethics Committee (Biomedical Investigation Ethics Committee of La Fe University Hospital of Valencia, Spain). Each patient signed an informed consent

prior the tissue collection. The study was conducted in consonance with the guidelines of the Declaration of Helsinki.

3.2. RNA extraction

Homogenization of heart tissue samples was performed using TRIzol® reagent in TissueLyser LT (Qiagen; Manchester, UK). RNA was extracted with PureLink™ Kit (Ambion Life Technologies; Carlsbad; CA, USA), following the protocol provided by the company. RNA concentration was measured with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific; Leicestershiere, UK). Microfluidics-based platform 2100 Bioanalyzer was used to determine RNA purity and integrity of the samples with the RNA 6000 Nano LabChip Kit (Agiletn Technologies; Santa Clara, CA, USA). All RNA samples displayed a 260/280 absorbance ratio ≥ 2.0 and reached a minimal RIN ≥ 9.

3.3. RNA-sequencing analysis

After RNA extraction, poly (A)-RNA samples were isolated from 25 µg of total RNA using the MicroPoly (A) Purist Kit (Ambion, Life Technologies, Carlsbad, CA, USA). Whole transcriptome libraries were obtained using SOLiD 5500 XL platform (Life Technologies; Carlsbad, CA, USA). The assay was performed from total poly (A)-RNA samples isolated, following company's recommendation. Quality of cDNA amplification was evaluated using the Bioanalyzer 2100 DNA 1000 Kit (Agilent Technologies; Santa Clara, CA, USA) and quantified using the Qubit 2.0 Fluorometer (Invitrogen; Paisley, UK).

SOLiD-templated beads were stablished using the whole transcriptome libraries, following guidelines provided by the company. 50625 pared-end protocol was used to sequence samples, producing 75 nt + 35 nt (pairended) + 5 nt (barcodes) sequences. Software from SOLiD Experimental Tracking was used to measure quality data obtained by the sequencing process (Gil-Cayuela et al., 2018).

3.4. RNA-sequencing data computational analysis

Paired-end reads obtained from sequencing, that represents the whole transcriptome, were mapped against latest version of the human genome (version GRchr37/hg19). Life Technologies mapping algorithm (http://www.lifetechnologies.com/; version 1.3) was used to achieve the total mapping. Alignments were expressed as BAM/SAM format files. Insufficient quality reads were eliminated with Picard Tool (http://picard.sourceforge.net/; version 1.83), those with a Phred score <10. Gene predictions were estimated with Cufflinks method and expression levels with HTSeq software, eliminating multimapped reads and only considering unique reads.

Several aspects were considered to normalize reads, such as depth of global samples, gene length and CG composition. To do so, edgeR method (version 3.2.4) was employed as differential expression analysis considering previous aspects. This method is based on a Poisson model, estimating variance of RNA-seq data for differential expression. Genes that show differential expression at a significance threshold of $p<0.05$ were selected. Data obtained from this assay was deposited in NCBI's Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number GSE55296 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55296).

3.5. Identification of mitochondrial dynamic genes

An exhaustive bibliography research was performed to identify molecules involved in mitochondrial dynamic processes: fusion, fission, mitophagy and cristae formation. These

procedures were analyzed from a molecular point of view, studying biological function of each protein and interactions stablished between each other, understanding how they could be involved in ICM predisposition. These molecules were obtained from bibliography research in PubMed and UniProt database, where molecular function and cellular location are detailed.

Identified molecules were organized in four categories according to their molecular function and implication in each mitochondrial dynamic process. Molecules that belong to their protein families were also studied in the computational analysis. Those implicated in mitochondrial processes were included in the final lists of genes.

3.6. Transmission electron microscopy

Alterations in mitochondrial structure were observed through TEM micrographs. $1mm^3$ sized LV samples were got into a solution of 1.5% of glutaraldehyde and 1% of formaldehyde in a 0.05M cacodylate buffer (pH 7.4) for 1h at 4°C. A post-fixation was assessed in buffered 1% OsO_4 for 1 hour at 4°C, then dehydrated in serial ethanol solutions and put into Epon 812 solution. Semi-thin and ultra-thin sections were obtained by Ultramicrotome Leica EM UC6. Semi-thin ones were evaluated with a light microscope (Olympus BX-50).

Then, ultra-thin sections, with a size of 80nm approximately, were mounted on cooper grids. They were counter-stained with 2% uranyl acetate for 20 minutes and 2.7% lead citrate for 3 minutes.After the processing of the samples, JEOL JEM-1010 microscopy system (Massachusetts, USA) was used to obtain micrographs with magnifications from x3000 - 12000. Before the obtention of final micrographs, different sections were inspected to avoid regions with artefactual changes or non-desired alterations (Portolés et al., 1994).

3.7. Statistical analysis

Data are presented as the mean ± standard deviations for continuous variables and as percentages for discrete variables. The Kolmogorov-Smirnov test was used to test variables for normal distribution. Between-group comparisons of tissue mRNA were performed using the Student's t-test (for variables with a normal distribution) or the Mann-Whitney U test (for variables with a non-normal distribution).

Fold change (FC) was obtained comparing control and ICM patients means. Patients with ≥ 1.3-fold were considered as altered genes. Genes with $p< 0.05$ were considered as statistically significant. All statistical analyses were performed using SPSS software v.20 for Windows (IBM SPSS Inc., Chicago, IL, USA).

4. RESULTS

4.1. Patients' clinical characteristics

A total of 13 ICM subjects were included in our population of study. All of them were men (100%) with a mean of 54 ± 8 years. These patients were classified as III-IV class in the New York Heart Association functional classification. Clinical characteristics of the ICM patients are summarized in Table 1.

Table 1. Clinical characteristics of ICM patients.

ICM patients	
n=13	
Age (years)	54 ± 8
Gender male (%)	100
NYHA class	3.5 ± 0.4
BMI (kg/m2)	26 ± 4
Total cholesterol (mg /dl)	162 ± 41
Prior hypertenson (%)	30
Prior diabetes mellitus (%)	38
Hemoglobin (mg/ml)	14 ± 3
Hematocrit (%)	41 ± 6
Duration of disease (months)	45 ± 40
Echo-Doppler study	
EF(%)	24 ± 4
LVESD (mm)	55 ± 7
LVEDD (mm)	64 ± 7
LVMI (g/m2)	139 ± 36

Data are expressed as the mean value ± standard deviation. NYHA, New York Heart Association; BMI, body mass index; EF, ejection fraction; LVESD, left ventricular end-systolic diameter; LVEDD, left ventricular end-diastolic diameter; LVMI, left ventricular mass index.

Samples for the CNT group were provided from non-diseased donors discarded for transplantation due to blood type or size incompatibility, derived from motor vehicle accidents or ictus patients. CNT group was mainly formed by men (80%) with a mean age of 47 ± 16 years. Clinical characteristics of the CNT group were not available for the study, in accordance with the Spanish Organic Law on Data Protection 15/1999.

4.2. Total studied genes

After a preliminary bibliography research in UniProt, a total of 96 genes were identified as related with mitochondrial dynamic processes. All their family genes were identified from RNA-seq database and checked individually by PubMed bibliography, incorporating 28 genes of these to the final study according to evidences about their implication in the processes. Finally, 124 genes were analyzed.

As shown in Table 2, a total of 10 genes were related with fusion, 18 genes with fission, 56 genes with mitophagy, and 40 genes with cristae formation process. These genes are detailed in Supplementary data, distributed in four categories according with their molecular function. In the appendixes, main characteristics of the genes are detailed, such as the name of the gene, NCBI ID, the protein location that they codify, molecular function according with the GO ontology and the bibliography each gene was obtained from. A total of 22 mitochondrial dynamics genes were determined as altered in ICM patients.

Table 2. Summary of total studied genes.

	STUDIED MITOCHONDRIAL GENES			ALTERED GENES	
	UniProt research	RNA-seq analysis and PubMed	Total of genes analyzed	Number (%)	Altered gene names
Mitochondria **fusion**	9	1	10	0	
Mitochondria **fission**	14	4	18	2 (11%)	***MTFR1L, RAB7L1***
Mitophagy	37	19	56	12 (21%)	*PARK2, CHDH,* ***BCL2L1****, BCL2L13,* ***BCL2L2****, RNF185,* ***USP36****,* ***MAP1LC3A****, SREBF1, CALCOCO2,* ***GABARAPL1, TBC1D17***
Cristae formation	36	4	40	8 (20%)	*ATP5D, ATP5G2, ATP5I, ATP5J2, ATP5L, C19orf70, APOO, ATPIF1*
Total	96	28	124	22 (18%)	

In studied mitochondrial genes section, genes are distributed regarding if they were obtained from UniProt or from RNA-seq and PubMed research. Altered gene names are detailed, considering a FC≥1.3 and p<0.05. Bold genes were obtained from RNA-seq database and checked in PubMed bibliography.

4.3. Fusion-related molecules

A total of 10 genes related with mitochondrial fusion were analyzed from RNA-seq data. All of them are detailed and referenced in Appendix 1 in Supplementary information. Main genes that are part of this group are mitofusins (*MTFN1, MTFN2)* and *OPA1*, that regulates this mitochondrial process. Mitofusins are embedded in OMM, as well as other mediators as *PLD6* or *GPAM. OPA1* is inserted in IMM, as *OMA1* and *YME1L.* None of these genes were determined to be altered in ICM patients compared with CNT group.

4.4. Fission-related molecules

18 genes were determined to be involved in mitochondrial fission process. All of them are listed in Appendix 2 in Supplementary information. DNM1L, FIS1 and MFF are the main proteins implicated in this mitochondrial process. These three proteins are in the OMM, as well as SMCR7 or MUL1. Other proteins, as RAB7L1 and INF2 are not in the mitochondria, but located in other parts of the cell, as cytoskeleton or endoplasmic reticulum, respectively; MTFR1L is a mitochondrial protein whose location is not clear nowadays.

MTFR1L (FC= 1.38; p<0.05) and *RAB7L1* (FC= -1.43; p <0.05) were identified as altered. Fold change of both genes are represented in Figure 1. *MTFR1L* is overexpressed in ICM patients, while *RAB7L1* is decreased compared with CNT group.

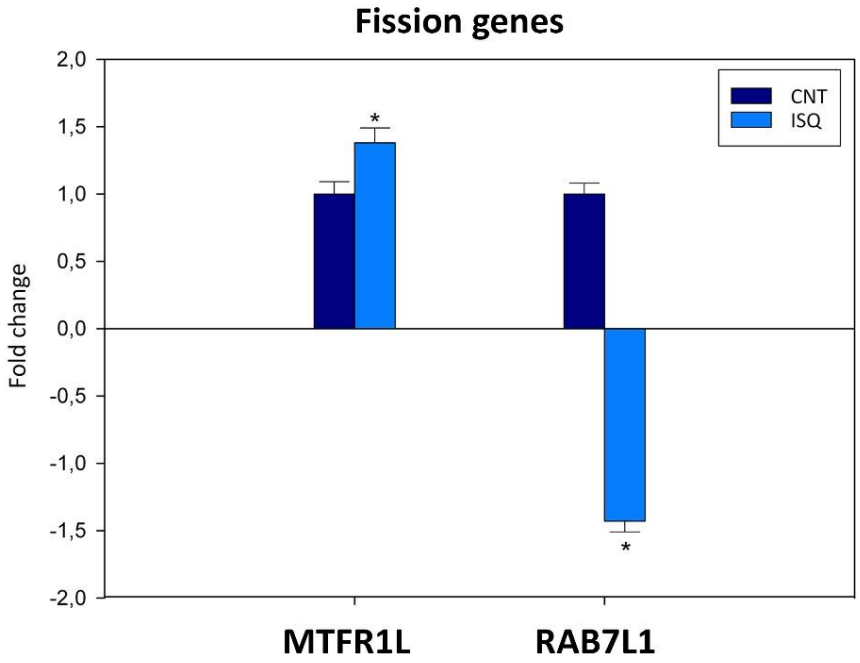

Figure 1. Altered Fission genes expression levels. Fold change ± SEM data of altered genes *MTFR1L* and *RAB7L1*. ICM data compared with CNT samples. Values of CNT group are set with a value of 1. *p<0.05.

4.5. Mitophagy-related molecules

56 genes were determined to be related with mitophagy process in mitochondria, as shown in Appendix 3 in Supplementary information. PINK1 and PARK2 are the main proteins that regulate mitophagy. They are located in both OMM and IMM, as well as CHDH, Bcl-2 family and RNF185. A high percentage of them are in other parts of the cell, such in cytosol (ULK1, SQSTM1, CALCOCO2*),* Golgi apparatus (SREBF1, SREBF2, WIPI1, WIPI2), endoplasmic reticulum (ZFYVE1, GABARAPL1) or autophagosome (MAP1LC3A, TBC1D17). Some of the genes codify ubiquitin proteins, such as USP30, USP36, MUL1, ATG12 or RNF185.

A total of 12 mitophagy genes were identified as altered. 6 genes are located out of the mitochondria, in the nucleolus, or in the cytosol; and the other 6 are found inside the mitochondria. Fold change of these altered genes are represented in Figure 2, divided according their location in the cell. *PARK2 (*FC= -1.49; p<0.05*), CHDH* (FC= -2.15; p<0.05*), BCL2L1 (*FC= -1.51; p<0.05*), BCL2L13* (FC= -1.38; p<0.01*), BCL2L2* (FC= -1.73; p<0.01) and *RNF185* (FC= -1.45; p<0.05) are localized inside the mitochondria (Figure 2A). All of them decrease their expression.

In summary, *USP36* (FC= -1.74; p<0.05), *MAP1LC3A* (FC= 1.67; p<0.05*), SREBF1* (FC= -2.03; p<0.01*), CALCOCO2* (FC= -1.62; p <0.0001), *GABARAPL1* (FC= -1.52; p <0.01) and *TBC1D17* (FC= 1.50; p<0.05) are located outside the mitochondria (Figure 2B). *MAP1LC3A* and *TBC1D17* are over-expressed in ICM patients, while the other non-mitochondrial mitophagy genes are under-expressed.

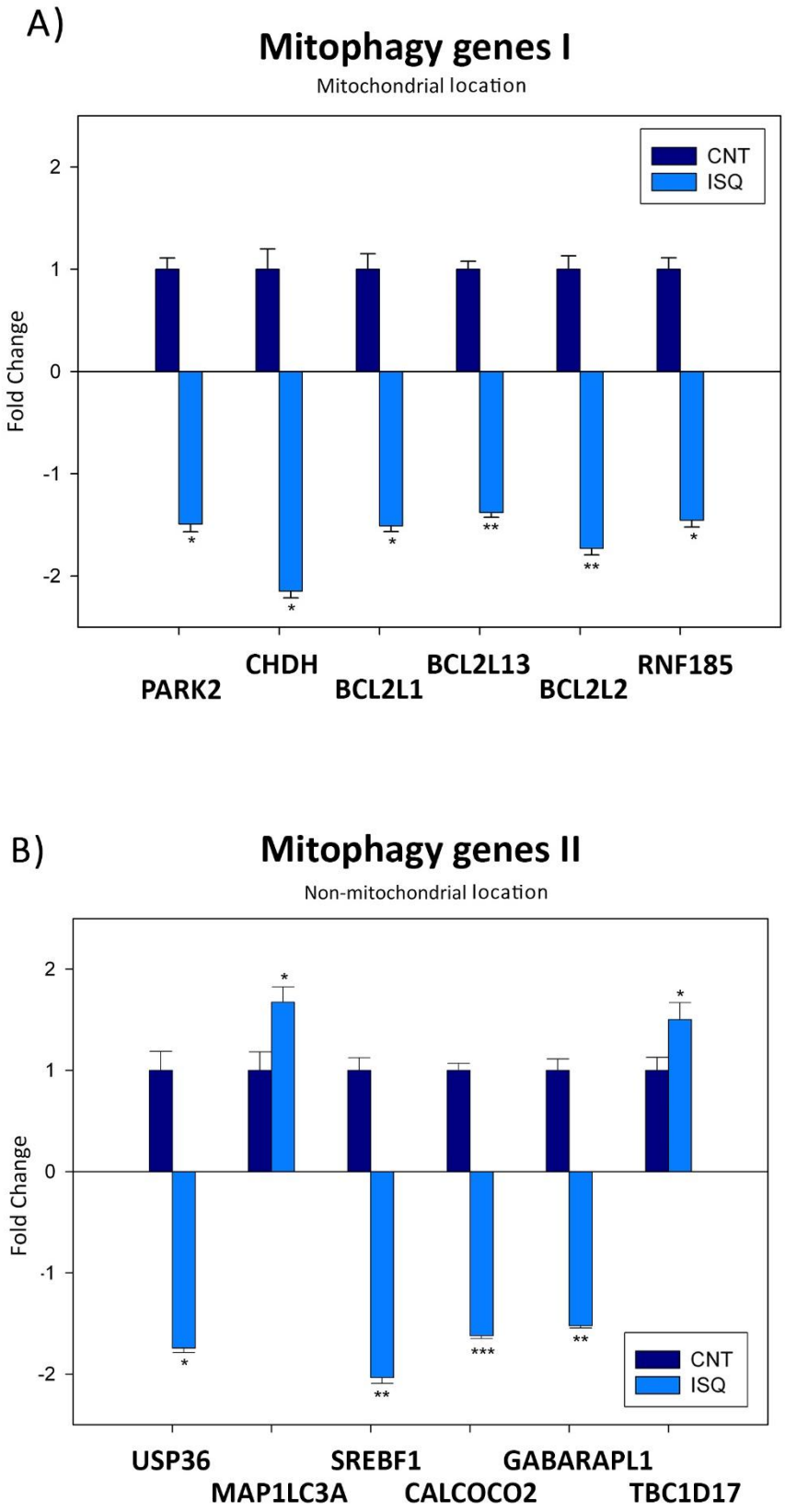

Figure 2. Altered expression of mitophagy genes. Fold change ± SEM of altered genes involved in mitophagy in ICM samples compared with CNT group. Values of CNT group were set to 1. *p <0.05, **p <0.01, ***p <0.001.

4.6. Cristae formation-related molecules

A total of 38 genes were detected as related with the process of cristae formation in mitochondria. All the proteins codified by these genes are embedded in the mitochondrial membrane, either in IMM or in OMM. In this category, genes are distributed by their implication in one of the two main complexes that are involved in cristae formation. 11 proteins are part of the MICOS complex, while 18 proteins form the ATP synthase complex or are linked to it. 9 of the genes related with this process are not part of any of these complexes, but they are in the mitochondrial membrane.

8 genes were determined to be altered in ICM patients comparing with CNT group. Fold change of each gene are represented in Figure 3. In ATPase complex, *ATP5D* (FC= 1.62; p<0.05*)*, *ATP5G2* (FC= 1.33; p<0.05)*, ATP5I* (FC= 2.04; p<0.01)*, ATP5J2* (FC= 1.47; p<0.05) and *ATP5L* (FC= 1.52; p<0.05*)* are altered in ICM patients (Figure 3A). The ATPase complex inhibitor *ATPIF1* (FC= 2.05; p<0.001) is also represented in Figure 3B. In MICOS complex, *C19orf70* (FC= 1.50; p<0.05) and *APOO* (FC= 1.65; p<0.01) are also altered (Figure 3C). All these cristae formation related genes are over-expressed in ICM patients comparing with CNT group.

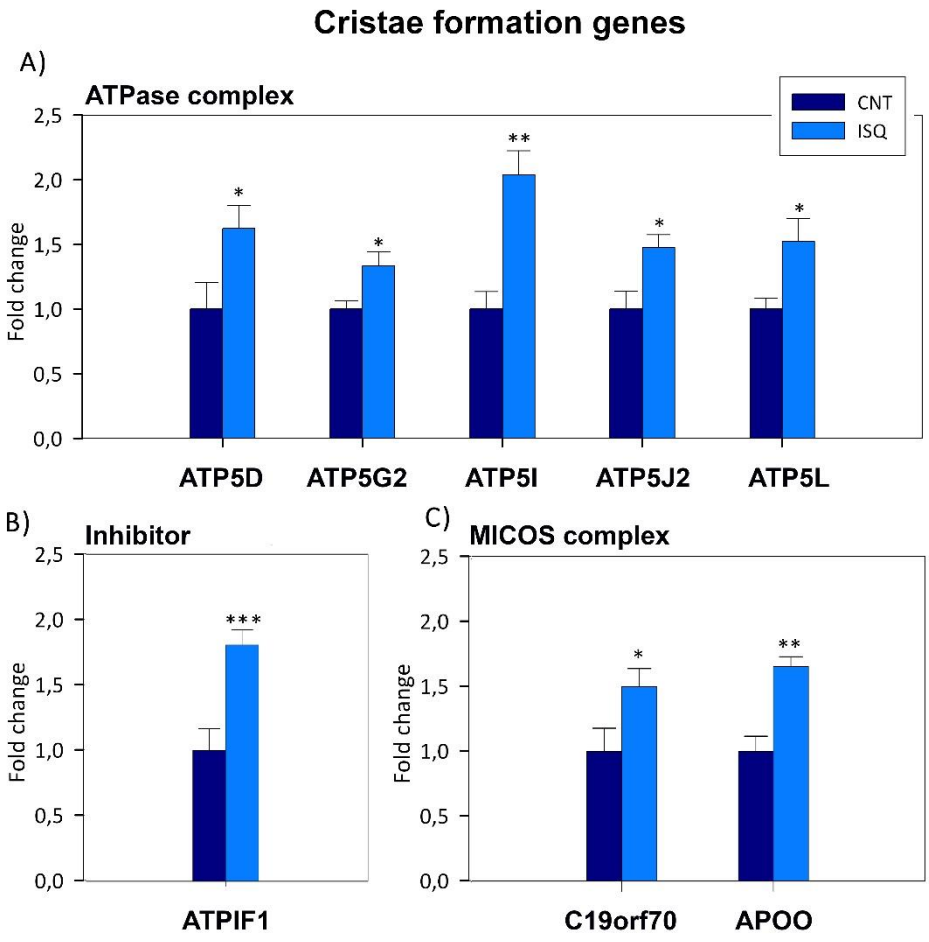

Figure 3. Altered cristae formation genes expression levels. Fold change ± SEM data of altered cristae formation genes distributed by their implication in the ATPase complex or in the MICOS complex. ICM samples were compared with CNT data. Values of the CNT group were set to 1. *p <0.05, **p <0.01, ***p <0.0001.

4.7. Transmission electron microscopy images

We studied a preliminary ultrastructural analysis to explore the potential changes in mitochondrial dynamics. Micrographs obtained from transmission electron microscopy (TEM) are presented in Figure 4.

In ICM, we found alterations in mitochondrial shape, size and distribution. These samples presented an irregular mitochondrial distribution and we also found alterations of sarcomeric units. In addition, mitochondria present a less electrodense matrix, probably caused by a swelling of the mitochondria, that derive in an anomalous mitochondrial function.

Although mitochondria membranes were in contact between them, none fusion or fission structures were observed. Matrix content was not shared between mitochondrial units that are in contact and intramitochondrial intermediate vacuoles were not observed, events that occur in fusion and fission processes. None autophagosomal structure were observed in the images. Cristae structures were not quantified in this study. Future steps are necessary to deepen in these preliminary results and to know if density of cristae structures is altered in ICM comparing with CNT.

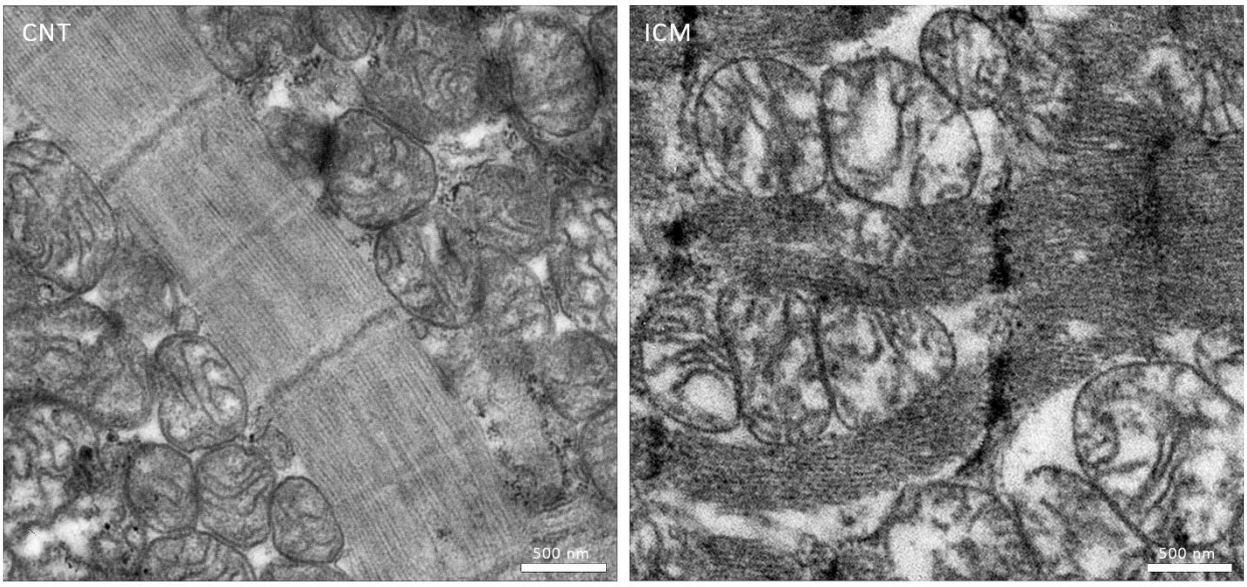

Figure 4. Micrographs of mitochondria in CNT and ICM patients. Left image shows mitochondrial structures in CNT tissue, while right image represents mitochondria location in ICM tissue. Scale-bar: 500 nm.

5. DISCUSSION

In this study, some mitochondrial dynamic genes are demonstrated to be altered in ICM hearts. Some genes related with fission, mitophagy and cristae formation processes were identified as altered comparing with CNT group, while none fusion-related genes were differently expressed in ICM patients. The identification of these cardiac alterations allows a whole interpretation of these processes in ICM and the implication of the genes in the development of this disease.

5.1. Fusion molecules

Mitochondria are high dynamics organelles under normal conditions (Van der Bliek et al., 2013). Fusion and fission processes establish a balanced system in order to maintain a high efficient quality control system of mitochondria health. Certain stress conditions affect the ratio between both processes in order to adapt mitochondria to new necessities. Some models of cardiac hypertrophy and HF evidenced that mitochondria fusion and fission events change under the effects of these diseases (Chen et al., 2009), but none study has demonstrated that these processes are altered in human ICM tissue.

A canine model was used to observe ultrastructural alterations in mitochondria after several brief occlusions, concluding that post-ischemic tissue suffered a decrease in the numerical density of the mitochondria and an increase in their volume, assuming that fusion events decreased in this situation (Pomar et al., 1995). Nevertheless, *Chen et al* demonstrated that mitochondria were smaller and more concentrated per area in hearts from rats that have suffered HF, suggesting an increased ratio between mitochondrial fission versus fusion in HF (Chen et al., 2009). Both results are contradictory among them, probably due to variability between tissue characteristics of each species. The results observed in TEM micrographs in this study do not reflect fusion events, according with our mRNA results.

Chen et al. demonstrated a decrease in OPA1 fusion-protein levels in human ICM patients, but not in mRNA expression of the gene that encoded the protein, according with our results. Also, they demonstrated that MTFN1 and MTFN2 fusion proteins in ICM human hearts. Results from *Chen et al.* were obtained only from Western Blot analysis, so conclusions are not comparable with the obtained in this study (Chen et al., 2009).

Based in our results, none fusion molecules were altered in ICM patients, so this process seems invariable in ICM patients.

5.2. Fission molecules

MTFR1L (mitochondrial fission regulator 1 like) belongs to MTFR1 family, a chondrocyte poly-proline region protein (also known as CHPPR) that promotes mitochondrial fission (Tonachini et al., 2002). MTFR1 family proteins are involved in aerobic respiration and mitochondrial organization, being embedded in IMM. Its poly-proline region has been demonstrated to regulate mitochondria fission by interacting with several proteins, but this process still remains unclear. Knockdown of MTFR1 in mice exhibits reduced mitochondrial fission, while MTFR1 transfected HeLa cells show spheroid mitochondria instead of tubular mitochondrial network (Monticone et al., 2010). In our study, *MTFR1L* was demonstrated to be overexpressed in ICM patients. Fission could be increased in these patients, but we cannot conclude that these processes occurs in the same way in mice and humans.

RAB7L1 *(*Ras-related protein Rab-7L1) is a GTPase regulator of vesicle trafficking, involved in the pathway of recycling proteins. This molecule is associated with some cytoskeleton structures, cell membrane and Golgi apparatus. RAB7L1*,* also known as RAB29, is part of the Rab32 subfamily proteins, involved in the regulation of DNM1L protein (Alto et al., 2002). DNM1L is one of the main proteins that regulates fission events in mitochondria, through oligomerization into membrane-associated tubular structures that wrap and cleave mitochondria. RAB71L regulates fission activity through the interaction with DNM1L (Ortiz-Sandoval et al., 2014). In our study, *RAB71L* was

demonstrated to be underexpressed in ICM patients, so DNM1L activity is less regulated and fission events are supposed to be higher.

Both molecules are involved in the regulation of fission process. These molecules were not classified in UniProt database as proteins involved in mitochondrial dynamics. After this study, these molecules could be defined as part of the molecular machinery of these processes. We can assume fission processes could not occur in higher percentage comparing with non-diseased hearts since we do not observe this phenomenon in this preliminary study.

5.3. Mitophagy

Autophagy is the main process that regulates the elimination of dysfunctional and damaged cellular components. Mitochondria are degraded by autophagosome in a process called mitophagy. Under stress conditions, autophagy is upregulated in order to eliminate damaged organelles and injured structures. When autophagy process is inefficient, several ROS species and useless cellular components accumulate in the cytoplasm and interfere in important cell activities. Damaged mitochondria consume large quantities of ATP while produce ATP below normal production levels (Gustafsson & Gottlieb, 2009).

Autophagy has been demonstrated to be upregulated in hypertrophic cardiomyopathy progresses to HF patients through endomyocardial biopsy and TEM images (Fidzianska et al., 2010). Also, as shown *Song et al.,* large quantities of early and late autophagic vacuoles with accumulated mitochondria residues and cytoplasmic remnants where observed in human explanted hearts with hypertrophy cardiomyopathy (Song et al., 2014). In dilated cardiomyopathy (DCM) patients, autophagy was shown to be upregulated, forming giant autophagosome ultrastructures enclosing mitochondria and other organelles of the cell (Saito et al., 2016). Several studies have demonstrated alterations in mitophagy activity in some cardiomyopathies, but none have described alterations ischemic human tissue.

Our results demonstrate that several mitophagy genes are downregulated. 10 genes are underexpressed of the total of 12 mitophagy genes altered. All the molecules located in mitochondria are downregulated. Some of them are main molecules involved in the process, such PARK2, that participate as regulator of the main mitophagy pathway in a cell, whose alteration is supposed to affect mitophagy in a high rate (Geisler et al., 2010). PARK2 receive mitochondrial damage signals through PINK1, that accumulates on the OMM of the damaged mitochondria and recruits PARK2, activating its ubiquitin ligase activity (Youle & Narendra, 2011).

Other molecules that are altered are part of the Bcl-2 family proteins (BCL2L1, BCL2L13, BCL2L2) that are involved in the regulation of apoptotic cell death (Tsujimoto, 1998). *CHDH* catalyzes the dehydrogenation of choline to betaine aldehyde in mitochondria and is involved in the activation of the PINK/PARK2 pathway (Park et al., 2014). *RNF185* is a ubiquitin that interacts with *BNIP1* and targets damaged mitochondria (Tang et al., 2011). All of them are located in the mitochondria and are downregulated in ICM patients.

Other molecules, that are located outside mitochondria, are also altered. GABARAP1 is part of the molecular mechanism of the autophagosome system, participating in later stage in autophagosome maturation (Chakrama et al., 2010). USP36 is a ubiquitin hydrolase that is involved in the selection of damaged organelles (Taillebourg et al., 2012). SREBF1 controls lipid homeostasis that regulates PINK/PARK2 pathway (Ivatt & Whitworth, 2014). CALCOCO2 interacts with

MAP1LC3A in the autophagosome structure (von Muhlinen et al., 2012). These molecules are downregulated in ICM patients.

Otherwise, only two molecules located outside the mitochondria were identified as upregulated: *MAP1LC3A* and *TBC1D17*, both located outside the mitochondria. MAP1LC3A is a ubiquitin-like modifier of the autophagosomal vacuoles that regulates autophagy process. MAP1LC3A interacts with GABARAP/GATE proteins to form autophagosome recognition unit that participates in the autophagy pathway recruiting damaged organelles tagged by adapter proteins (Yoshii & Mizushima, 2015). TBC1D17 *is* a GTPase protein that regulates negatively autophagy interacting with *Rab8* protein, that controls trafficking processes in the autophagy process. *TBC1D17* inhibits endocytic trafficking of transferrin receptor blocking Rab8 (Vaibhava et al., 2012).

In summary, we have demonstrated that mitophagy process is be downregulated in ICM patients. However, these data are obtained from mRNA levels, so they do not reflect post-translational modifications. Nevertheless, alterations involve main molecules of mitophagy, so this process could be downregulated in ICM hearts. Less mitophagy could lead to an accumulation of damaged and afunctional mitochondria, an accumulation of ROS species and an alteration in ATP production and consume.

5.4. Cristae formation

Cristae are the main mitochondrial structure involved in the production of ATP through the activity of the electron transport chain (ETC). The number of cristae is proportionate to the metabolic activity of the cell, so very metabolically active and dynamics cells, such cardiomyocytes, contains large numbers of cristae in their mitochondria. Several proteins are embedded or associated with this structure and alterations in the levels of these molecules may affect considerably to the mitochondrial activity.

Alterations in cristae morphology, number and structure were observed in HF left ventricle rat tissue by Liu et al 2014, showing loss of the normal mitochondria structure compared with CNT group. Also, they demonstrated a considerable reduction in Complex I activity, consistent with decreased mitochondrial respiratory function (Liu et al., 2014). Nevertheless, none study has research about alterations in cristae structures in human cardiac tissue.

As presented in Results, molecules involved in the cristae formation process are categorized according with their implication with two different complexes: ATP synthase and MICOS complex. All the altered genes involved in the cristae formation process were detected as upregulated. Thus, the alteration of both complexes in mitochondria of ICM patients could be a relevant finding.

ATP synthase complex, or Complex V, is part of the ETC, producing ATP from ADP in the presence of a proton gradient across the membrane. This complex is a molecular motor because it is an ATP generator and a key regulator of mitochondrial function (Long et al., 2015). ATP synthase complex is also involved in generating mitochondrial cristae morphology, as demonstrated by Davies et al (Davies et al., 2012). Complex V is the only complex that form the ETC that is involved in cristae formation and mitochondrial morphology.

As part of the complex ATP synthase, different genes were detected to be upregulated (*ATP5D, ATP5G2, ATP5I, ATP5J2, ATP5L)* in this study. All of them participate in the production energy through the transformation of ATP from ADP. Thus, alterations of these molecules imply both an

alteration in the energy production and in the cristae organization. An upregulation of this molecules in ICM patients could suppose an increase of the ATP production and cristae subunits.

ATPIF1 is an inhibitor of the ATPase activity of the Complex V that maintains mitochondrial membrane potential. As demonstrated by Campanella et al., *ATPIF1* overexpression leads to an increase of mitochondrial cristae (Campanella et al., 2008). In this study, *ATPIF1* was demonstrated to be overexpressed in ICM patients, so an increase of mitochondrial cristae structures is expected in these patients.

MICOS complex regulates IMM invaginations that finally form cristae structures. This complex is crucial for the formation and maintenance of cristae structure (Kozjak-Pavlovic, 2017). APOO, also known as MIC26, is a lipoprotein involved in maintaining cristae junctions (Koob et al., 2015). C19orf70, also known as MIC13, is involved in the maturation of the MICOS complex and the maintenance of cristae morphology (Anand et al., 2016). Both molecules are overexpressed in ICM patients compared with CNT group. An increase of the presence of both molecules is related with a major number of cristae junctions, leading to high stablished cristae structures.

In summary, altered cristae formation related genes were all overexpressed in ICM patients, so an increase of cristae structures in mitochondria in these patients could be relevant in the study of the physiopathology of ICM. ATPase and MICOS complexes genes were the most altered in this dynamic.

5.5. Limitations of the study

A common limitation of studies in which the cardiac tissues used are obtained from end-stage failing human hearts is that disease aetiology and treatments vary considerably. However, aetiologically homogenous population was ensured in that study population. Moreover, tissue samples were obtained from the transmural LV apex. Thus, the results and conclusions obtained in this study cannot be generalized to all layers and regions of the LV. However, a large number of samples collected from explanted human hearts with ICM were used in the study, in order to decrease as much as possible variability between samples. All samples were obtained from patients who had undergone cardiac transplantation, which makes our results applicable to the ICM population.

5.6. Future steps

This study evaluates mRNA levels through RNA-seq and statistical analysis, in order to detect which molecules involved in mitochondrial dynamics are altered in ICM patients. Thus, post-translational modifications and protein levels are not evaluated in this study, so the actions performed in this work are the first steps of a more complex project. Consequently, Western Blot assay will be performed to detect protein levels and immunofluorescence and immunocytochemistry assays will be assessed to localize altered proteins in cardiac cells.

More micrograph images are necessary to carry out a more complete study of the relation between altered genes involved in these processes and mitochondrial structures, especially in cristae density. A stereology interpretation of the images from all the patients will be carried out to obtain final conclusions.

6. CONCLUSIONS

1. In this study, four mitochondria dynamic processes were analyzed (fusion, fission, mitophagy and cristae formation) in human explanted heart tissue from ICM patients, showing alterations in 22 genes.

2. In fusion and fission mitochondrial processes, only two regulatory genes (*MTFR1L* and *RAB7L1)* of the fission dynamics modify their expression comparing with CNT.

3. Our results indicate that mitophagy and cristae formation are the dynamic process with the most relevant changes. Mitophagy genes located in mitochondria were all downregulated, (*PARK2, CHDH, PARK2, BCL2L1, BCL2L13, BCL2L2* and *RNF185*) while some genes located outside mitochondria were underexpressed (*USP36, SREBF1, CALCOCO2* and *GABARAPL1)* and only two of them were overexpressed (*MAP1LC3A* and *TBC1D17)*. The genes involved in cristae formation process were all upregulated, such the ATPase complex (*ATP5D, ATP5G2, ATP5I, ATP5J2* and *ATP5L),* the inhibitor of these complex *(ATPIF1)* and the MICOS complex (*C19orf70* and *APOO)*.

4. The changes in mRNAs related to mitophagy and cristae formation processes could increase damaged of the cardiac mitochondria in ICM, interfering critical activities of the cardiomyocyte, such as alterations in the ATP production that is necessary to a correct cardiac ventricular function.

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