UNIVERSITA' DEGLI STUDI DI UDINE

Department of Medical and Biological Sciences

PhD Course in Clinical Sciences and Technologies

XXVII Cycle

Autophagy And Inflammation In The Pathogenesis of Idiopathic Dilated Cardiomyopathy

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Academic Year 2015-2016

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1 - ABSTRACT

Background: Idiopathic dilated cardiomyopathy (iDCM) is a disease of cardiac muscle characterized by dilatation, in particular of left ventricle, and systolic dysfunction in absence of other pathological conditions note and it it is burdened by a serious morbidity and mortality. Recent literature data indicate that the loss of proteostasis is an important pathophysiological mechanism.

Aims: Starting from the observation that proteins aggregate as amyloid accumulations in cardiomyocytes of patients affected by iDCM, it was evaluated the hypothesis of a defect in the ubiquitin-proteasome system (UPS). It has been shown that aggresome stimulates an increase of autophagic flow so we wanted to assess the presence of elements indicative autophagy-lysosomal pathway (ALP) alterations. For this reason, we analyzed the presence of punctae of LC3 and levels of p62. An effect of the arrest of the autophagic/lysosomal pathway follows the accumulation of dysfunctional mitochondria within the cell. We evaluated also the accumulation of mitochondria positive to Parkin1, suggesting a defect in the removal of dysfunctional mitochondria.

Methods and Results: we compared 48 hearts of patients affected by iDCM, collected at the time of transplant, with 18 control hearts. We studied autophagic flux alteration, mitochondrial dysfunction, activation of the damage response to double-stranded DNA, inflammasome activation, NF-*k*B activation and myocyte hypertrophy. Furthermor we compared cardiac stem cells obtained by explanted hearts of iDCM patients and donors studying senescence, proliferation and lysosomal membrane permeabilization.

Conclusions: data obtained suggest that patients affected by iDCM present a vicious cycle characterized by loss of proteostasis, defects of autophagic flux, accumulation of dysfunctional mitochondria, increase of free intracellular radical, activation of the cellular DNA damage response, activation of inflammatory response with further stimulus to myocyte hypertrophy and worsening proteostasis.

2 - INTRODUCTION

2.1 Human Heart Anatomy

The heart is a muscular organ that, with its rhythmic contractions, determines and maintains blood circulation. It is divided into two parts, right and left, separated from each other. Each half is in turn formed by two communicating rooms, the upper, said atrium, which receives blood from the periphery, and the lower, said ventricle, which pushes the blood towards the periphery.
The heart is located in the chest cavity, a space between the two lungs (mediastinum), placed on the phrenic center of the diaphragm. In the thoracic cavity it is moved anteriorly and to the left. It is protected, forward, sternum and costal cartilages, rear corresponds to the thoracic vertebrae from the fifth to the eighth and top extends to the upper opening of the chest through the vascular pedicle, formed by great vessels originating and ending in the heart (ascending aorta, pulmonary trunk and superior vena cava).
It has a conical shape and is slightly flattened in the anteroposterior direction. The tip, or apex, turns down, forward and to the left, while the base is in the upper and looks to the right and back. The heart weighs about 250-300g and contains about 500 ml of blood. It is totally immersed in the pericardium, a serous membrane that forms a double bag, wet internally by a veil of pericardial fluid.
The atria communicate with the ventricles below through two orifices provided with valves: the tricuspid valve to the right and the mitral valve to the left. The consistency of the myocardium is different in the two rooms: appears greater in the ventricles, which have pumping action, where the tissue is more robust while is thinner in the walls of the atria.
In the right atrium flow into three veins: the inferior vena cava, the superior vena cava and the great coronary vein; in the atrium of the left, instead, the pulmonary veins that carry oxygenated blood. The pulmonary artery origins from the right ventricle, while the aorta origins on the left side. Also the orifices between ventricles and arteries, as the orifices atrioventricular, are occupied by valve devices: the aortic valve and the pulmonary valve. The heart valves are made up of fibrous sheets (cuspids) inserted in the rings fibrous skeleton of the heart; those which are located between the atria and ventricles are constituted by the tricuspid and mitral right to left.

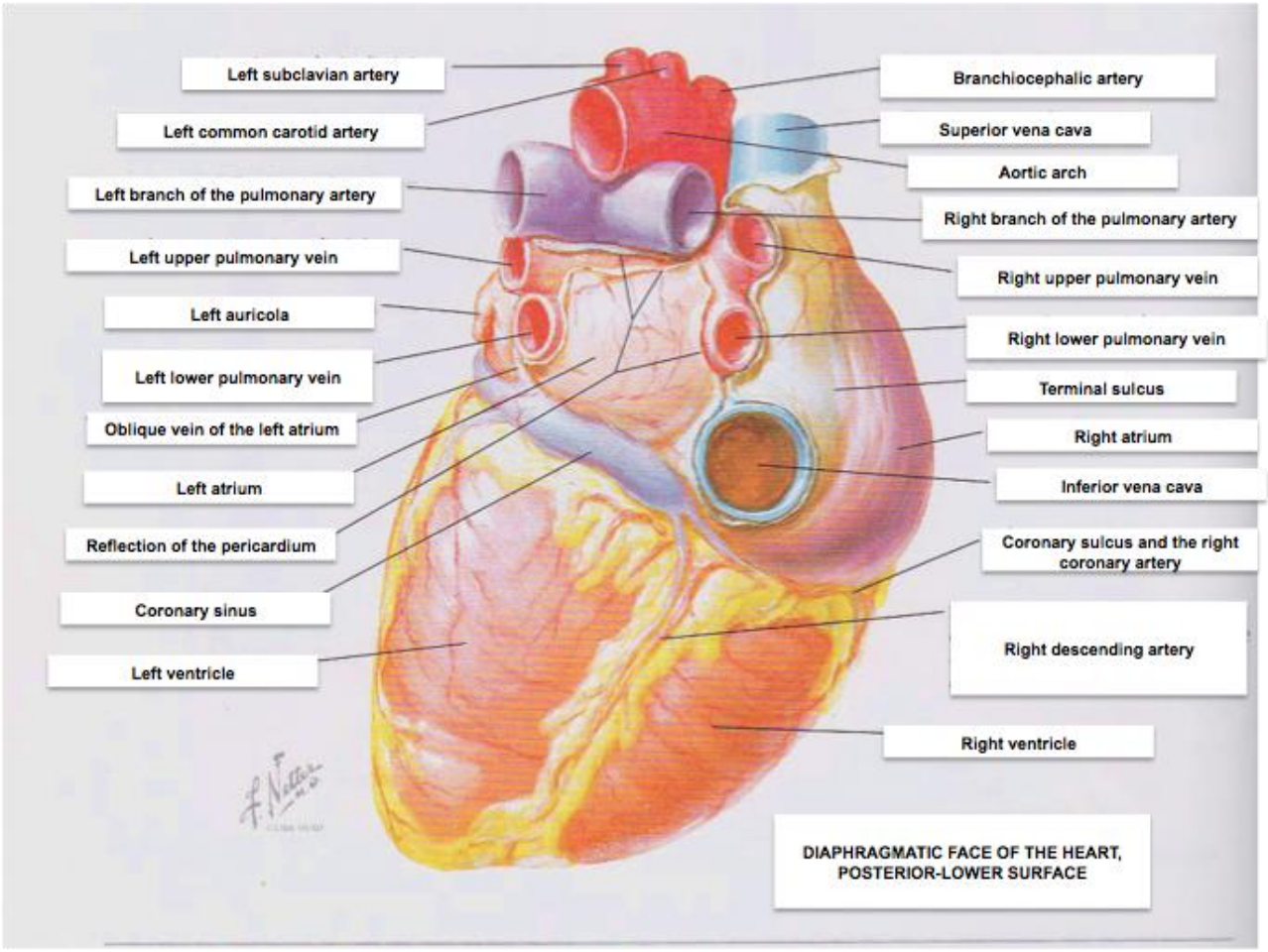


Figure 2.1 Diaphragmatic face of the heart, posterior-lower surface. Image taken and modified from F.H. Netter. Atlante di Anatomia Umana - Novartis Edizioni, 1998.

The heart is composed mainly of muscle (myocardium) and its rhythmic and autonomous contractions, on average 60-70 beats per minute in the subject at rest, ensures the circulation of blood in the vessels; with each pulse corresponds to a contraction phase, called systole, and a phase of relaxation, called diastole.
It can define the heart muscle as a hybrid of the skeletal muscle and smooth muscle, because the heart has a frequency and a contractility regulated by the nervous system and the intrinsic rhythm and a capacity of spontaneous contraction.
The heart wall is constituted by three distinct layers, respectively from the inside outward: endocardium, myocardium and pericardium. The endocardium, consisting of a layer of simple squamous endocardial cells, covers the inner surface of the heart. The myocardium (by *Myo* muscle and *Kardia* heart) is constituted by layers of cardiac muscle tissue, connective tissue, blood vessels and nerves. Muscle tissue is made up of cardiomyocytes.
The cardiomyocytes, responsible of the electrical and the contractile activity, are big cells (10-20 μm in diameter and 50-100 μm in lenght) which constitute the 75% of the myocardium volume and 1/3 of total cardiac cells. The intercalated disks are very important for activities of cardiomyocytes. They are specialized regions of intercellular contact, where he wings adherens, desmosomes and gap-junction are located, which provide for a coupling both electrical and mechanical, allowing the passage of ions and the transfer of the voltage contractile from one cell to another. The cells, with these close links, contract almost simultaneously forming the so-called "functional syncytium". The connective tissue of the heart is produced by cardiac fibroblasts, the only cell type cardiac not to be associated to the basement membrane. These are cells with mesenchymal characteristic, mainly originated by pro-epicardic organ and specialized in the production

of wide variety of extracellular matrix components such as collagen, mucopolysaccharides, elastic fibers, reticular and glycoproteins and biochemical mediators such as growth factors and proteases. This tissue has function of support for the cardiomyocytes, the vessels and the nerves of the myocardium.
The epicardium, the serosa of visceral pericardium that covers the outer surface of the heart, is constituted by a layer of simple squamous mesothelial cells that covers a layer of connective tissue support.

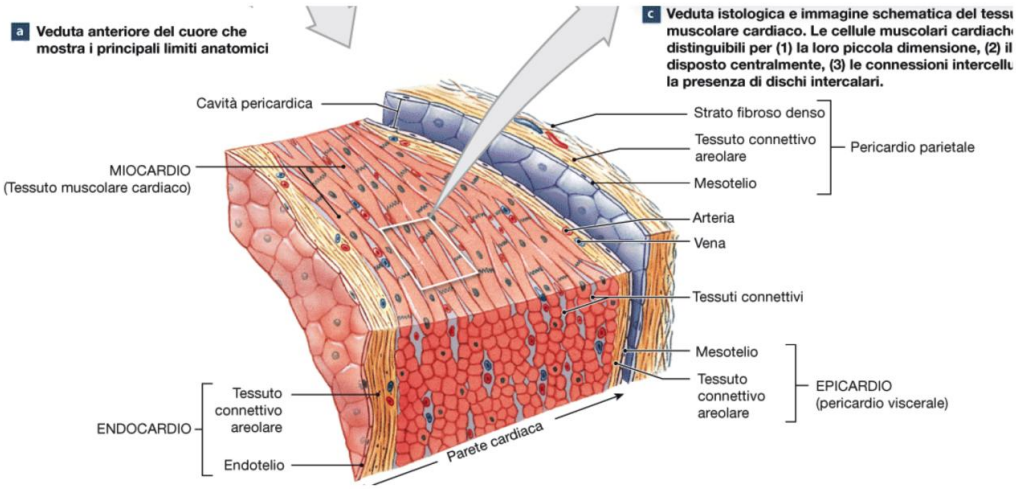


Figure 2.2 Heart wall section which show epicardium, myocardium and endocardium. Taken from: Martini, Timmons, Tallich. Anatomia Umana; Edises 2013.

2.1.1 Cardiovascular Diseases

Cardiovascular disease (CVD) (e.g., peripheral vascular disease, coronary heart disease, stroke, and myocardial infarction) is a main cause of death in both undeveloped and developed countries [1]. According to the American Heart Association in 2015, the prevalence of total coronary heart disease is 6.2% in adults (age ≥ 20) [2]. The cardiovascular diseases are a heterogeneous group that includes: cardiac diseases, cerebrovascular diseases and blood vessel disease.
There are two main group of cardiovascular disease: 1) disease due to atherosclerosis, such as coronary artery disease, cerebrovascular disease, diseases of the aorta and the arteries; 2) other cardiovascular disease as congenital heart disease, rheumatic heart disease, cardiomyopathy and cardiac arrhythmias.
Obesity, cigarette smoking, hyperlipidemia, hyperglycemia, and a sedentary lifestyle are typical risk factors associated with CVD so the majority of cardiovascular diseases can be prevented eliminating these risk factors [3].
People with cardiovascular diseases, or with high cardiovascular risk for the presence of one or more risk factors such as family history, hypertension, diabetes, hyperlipidemia, require a rapid and early diagnosis.

2.1.2 Heart Failure

Heart function fails when the organ is unable to pump blood at a rate proportional to the body's need for oxygen or when this function leads to elevated cardiac chamber filling pressures (cardiogenic pulmonary edema).
The prevalence of heart failure is 2.5% of the US population or 5 million patients (from the National Health and Nutrition Education Survey) [4].
Common etiological mechanisms of heart failure include coronary ischemia, valvular disease, hypertension, and diastolic dysfunction. Yet, other causes include: postpartum cardiomyopathy, chronic tachycardia, metabolic dysregulation, adverse medication side effects (particularly adriamycin chemotherapy), Duchenne's muscular dystrophy, infiltrative diseases (such as amyloidosis), and inflammatory/connective tissue diseases (such as systemic lupus erythematosus). When known causes of heart failure are excluded, then heart failure is classified as idiopathic. There also cases in which acute decompensated heart failure is associated with abrupt-onset symptoms associated with hospitalization. Nearly half of the admitted patients with heart failure have preserved ejection fraction. [4,5].

It was shown that heart failure is a process ingravescent and potentially irreversible, whose progression is accompanied by a series of structural alterations that takes the name of remodeling.

2.1.3 Sudden Cardiac Death

Sudden death (SD) is defined as a "*natural and unexpected event that occurs within the first hour after onset of symptoms in an apparently healthy subject or whose disease was not severe enough to predict a fatal outcome, and in which a thorough postmortem examination fails to demonstrate an adequate cause of death*" [6]. Nearly 85% of all SD are of cardiac origin, called sudden cardiac death (SCD). SCD is a leading cause of death in Western countries, and despite a huge number of deaths it remains without a definitive diagnosis [7].

Cardiology has included advances in genetics, identifying several new genes responsible for disease associated with SCD. As a consequence, genetic testing has been progressively incorporated into clinical diagnoses, identifying the cause of the disease in clinically affected patients, unresolved post-mortem cases and even in asymptomatic individuals who are at risk of SCD because they carry the genetic alteration responsible for the disease [8].Therefore, genetics has been incorporated into current clinical guidelines on SCD.

Although physical activity is considered an important factor for the primary and secondary prevention of cardiovascular disease, exercise faced by athletes with silent and unrecognized disease is a risk factor for sudden cardiac death [9]. The most common cause of sudden death is heart disease, followed by structural abnormalities and arrhythmogenic conditions predisposing to heart disease [9].

2.2 CARDIOMYOPATHIES

2.2.1 Definition

Cardiomyopathies (CMPs) are myocardial disorders in which the heart muscle is structurally and functionally abnormal in absence of conditions such as coronary artery disease, hypertension or valvular disease, sufficient to cause the observed myocardial abnormalities [10].

Cardiomyopathies are classified traditionally according to morphological and functional criteria into four categories: dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM) and arrhythmogenic right ventricular (RV) cardiomyopathy/dysplasia (ARVC/D). DCM is the most common form of heart muscle disease, comprising approximately 60% of all cardiomyopathies and characterized by left ventricular (LV) dilation and systolic dysfunction. The dilated cardiomyopathy is often assumed as a common pathway of several cardiovascular pathologies [11].

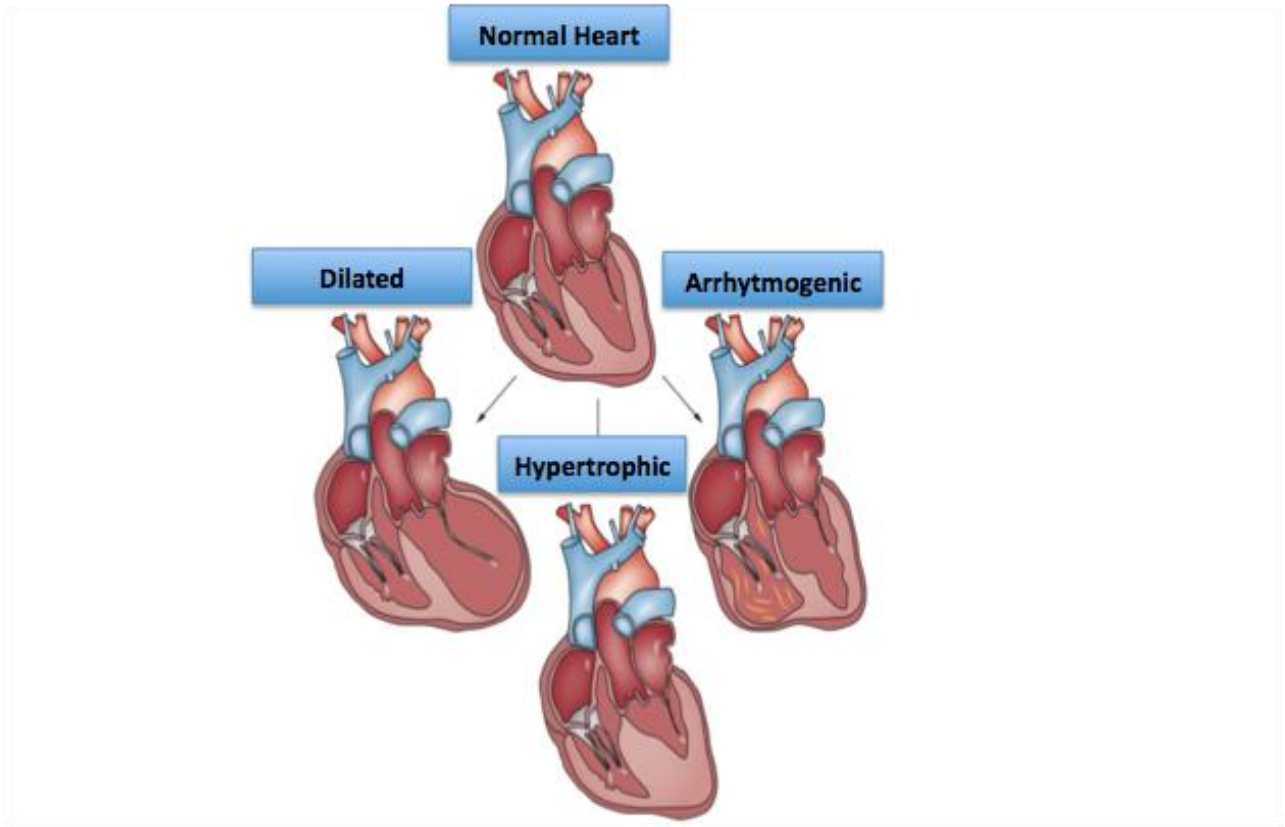


Figure 2.3 Phenotypic Classification of cardiomyopathies. Taken from: Hershberger R.E., Hedges D.J. & Morales A. (2013). **Dilated cardiomyopathy: the complexity of a diverse genetic architecture.** Nature Reviews. Cardiology, 10(9), 531-47.

Cardiomyopathies are classified as either primary or secondary. Primary cardiomyopathies consist of disorders namely or predominantly confined to the heart muscle, which have genetic, nongenetic, or acquired causes. Secondary cardiomyopathies are disorders that have myocardial damage as a result of systemic or multi-organ disease [12]. These cardiomyopathies can be primary myocardial disorders or develop as a secondary consequence of a variety of conditions, including myocardial ischemia, inflammation, infection, increased myocardial pressure or volume load and toxic agents.

In the 1980 World Health Organization (WHO) classification, cardiomyopathies were classified as “heart muscle diseases of unknown cause, reflecting a general lack of

etiologic factors which may cause heart failure. The next WHO classification published in 1995 proposed “diseases of myocardium associated with cardiac dysfunction” and included for the first time ARVC/D, as well as primary RCM [13].

A more recent definition and classification of cardiomyopathies was proposed by the American Heart Association (AHA) Scientific Statement Panel. The AHA divides cardiomyopathies into two major groups based on predominant organ involvement. Primary cardiomyopathies (genetic, nongenetic, or acquired) are those solely or predominantly confined to heart muscle and are relatively less common. Secondary cardiomyopathies show pathological myocardial involvement as part of a several number of systemic pathologies [14].

In 2013, the MOGE(S) classification for a phenotype-genotype nomenclature of cardiomyopathies was proposed by the World Heart Federation [15]. This classification suggests a nosology that addresses five characteristics of cardiomyopathic disorders: morphofunctional state (M), organ involvement (O), genetic inheritance (G), etiologic annotation (E) and functional state (S) according to ACC/AHA A-D stage and New York Heart Association (NYHA)I-IV functional class. The description of five characteristics provides classification in MOGE(S) designation. The MOGE(S) classification has several advantages with regard to simultaneous maximal description of disease from clinical and genetic points. However, this classification does not fulfill the diagnostic criteria of cardiomyopathies in several clinical situations and may not be always applied in clinical practice, because of the lack of genetic testing in many clinical centers. On the other hand, the classification based on systematically genetic testing and monitoring may cause overdiagnostic states without clinically evident signs of cardiomyopathies and absence of clinical phenotype. Further genetic research and development of multicenter registries are needed to clarify the clinical advantages and to make more practical of MOGE(S) classification of cardiomyopathies.

It is important the substantial genetic overlap observed between DCM and HCM and the genetic and phenotypic overlap between DCM, HCM and ARVC [16].

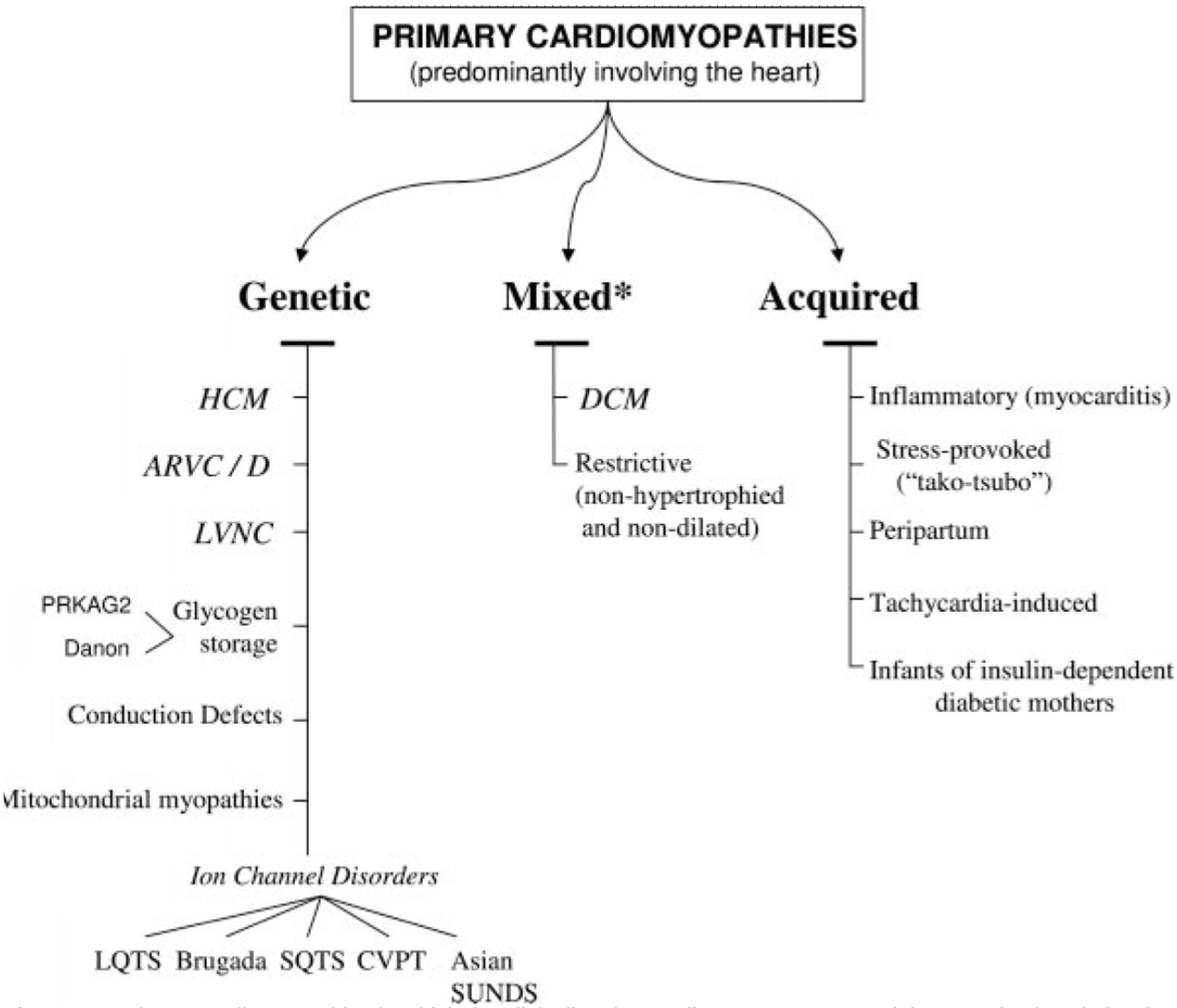


Figure 2.4 Primary cardiomyopathies in which the clinically relevant disease processes solely or predominantly involve the myocardium. The conditions have been segregated according to their genetic or nongenetic etiologies. *Predominantly nongenetic; familial disease with a genetic origin has been reported in a minority case. Taken from: Maron B.J., Towbin J.A., Thiene G., Antzelevitch C., Corrado D., Arnett D., Moss A.J., Seidman C.E., Young J.B. **Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention.** Circulation (2006), 113(14):1807-16.

2.2.2 Dilated Cardiomyopathy

2.2.2.1 Epidemiology and Genetic

Dilated cardiomyopathy is a relatively common but poorly understood group of disorders that result in heart failure and premature death [17]. It is a heart muscle disease characterized by dilation and systolic dysfunction in absence of other pathologic conditions known enough to cause a global contractile dysfunction. This condition is burdened with high morbidity and mortality (20% at 5 years) as consequence of cardiac failure and sudden death [18]. Although ischemic, toxic, metabolic, or infectious causes are recognized, inherited gene defects account for 25 to 30 percent of cases [19]. Of the five mutated genes known to cause dilated cardiomyopathy, four are often associated with additional clinical manifestations. Among these, defects in the cytoskeletal proteins

dystrophin [20] desmin, [21] and tafazzin [22, 23] produce both myocardial and skeletal-muscle dysfunction, whereas lamin A/C mutations [24] cause ventricular dysfunction with conduction-system disease. It is important also the role of titin gene that, according to the latest estimates, it would be changed in 25% of family forms and in 13% of the forms not selected [25]. Familial dilated cardiomyopathy that is unaccompanied by other clinical manifestations is a heterogeneous disorder [26], and although disease loci have been defined on chromosomes 1q32 [27], 2q11–22 [28], 2q31 [29], 9q13–22 and 10q21–23 [30] only two disease-causing mutations have been identified, both in the cardiac actin gene.[31]. The expressiveness of DCM is extremely variable, resulting in individuals carrying the same mutation, paintings ranging from mild ventricular dysfunction to overt heart failure [32].
Also the penetrance of DCM is variable and is linked to the age of the patient: it manifests in subjects aged between 30 and 60 years (although it can occur in childhood and neonatal).

2.2.2.2 Histology and Fisiopathology

The main alteration in dilated cardiomyopathy is constituted by the inefficient contraction. Patients may present an ejection fraction below 25% (normal 50 to 65%). Consequently, it is observed a progressive heart failure refractory to therapy. An exception to this unfavorable course, is given by peripartum cardiomyopathy in which about half of patients experience spontaneous healing [33].
Idiopathic Dilated Cardiomyopathy is characterized by a progressive dilation of ventricular cavities associated with thinning of the myocardial wall. This feature can also be a consequence of a series of complex structural alterations, cellular and molecular which mark the evolution of a broad spectrum of pathological conditions. This evolution is known as cardiac remodelling [34].

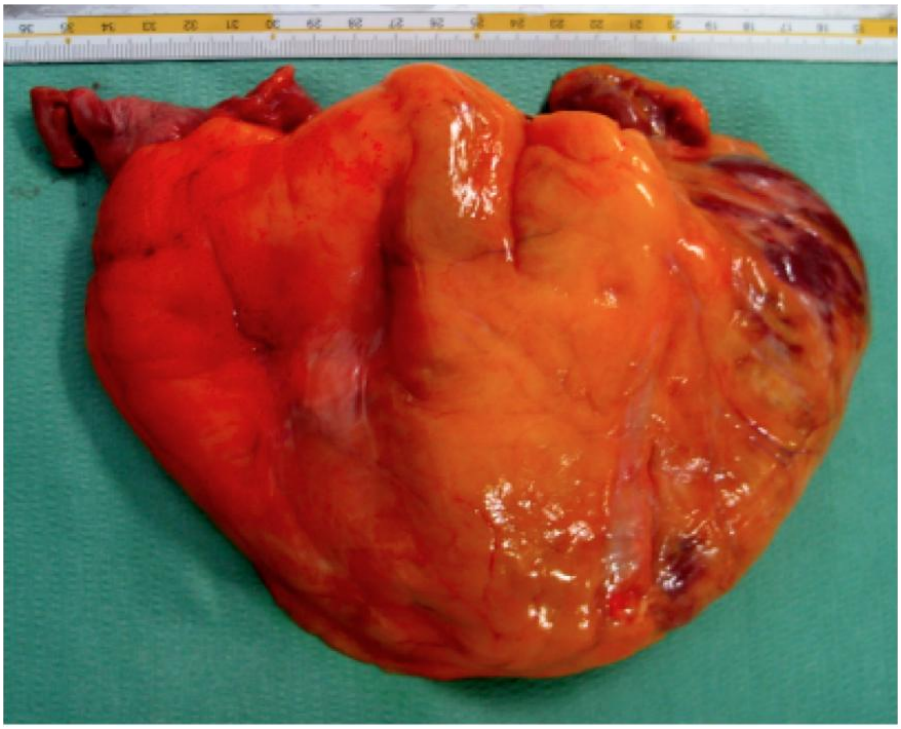


Figure 2.5 Heart explanted by a patient affected by dilated cardiomyopathy
A patients affected by DCM can present:

• The heart is globally larger and the cavities are dilated, with walls initially normal thickness, but which, with the passage of time, become thinner gradually
• The valvular and coronary arteries are anatomically normal.
• Histologically there are nonspecific alterations, commonly found in other situations characterized by major ventricular dilatation.
• Marked biventricular dilation with thinning of the walls

Morphometric studies conducted since the 90s have shown that, although the DCM is characterized by foci of fibrosis replacement (<1 cm2) and diffuse interstitial fibrosis, it is very rare to observe large areas of fibrosis. The same studies have demonstrated that although an observed reduction in the volume fraction occupied by myocytes (due to the increase of fibrosis), this is accompanied by myocyte hypertrophy due mainly to the increase in myocyte length. Finally, these alterations are associated with reduction in the number of myocytes that make up the wall thickness. Taken together, these observations support the hypothesis that ventricular dilatation is due to the combined effect of lengthening miocitario and sliding against each myocytes [35].
Fibrosis is an independent prognostic predictor, also associated with sudden cardiac death (6), while the study of cellular and molecular mechanisms, associated with contractile dysfunction and cavity dilation, is more complex. Different authors have observed a relationship between ventricular dilation, wall thinning, as a result of increased wall stress (according to the law of Laplace; $\sigma = PR / 2h$, where σ = wall stress, P = pressure intracavitary, R = radius cavitary, h = wall thickness) and activation of systems of mechanotransduction, including the local renin angiotensin system, resulting in cardiomyocyte death.

2.3 STEM CELLS: DEFINITION AND CLASSIFICATION

The term “Stem Cell” was firstly proposed together with the existence of haematopoietic stem cells by Alexander Alexandrowitsch Maximow in 1908 [36], and it indicates immature precursor cells with the self-renewal ability and the great potential of multilineage differentiation [36].
A cell to be recognized as a stem cell has to satisfy four criteria [37, 38]:

1. Stem Cells undergo multiple, sequential self-renewing cell divisions, a prerequisite for sustaining the population;
2. Single stem cell-derived daughter cells differentiate into cells that have different, more restricted properties;
3. Stem cells functionally repopulate the tissue of origin when transplanted in a damaged recipient;
4. Stem cells contribute differentiated progeny in vivo even in the absence of tissue damage.

Depending on their differential potential, stem cells are classically grouped as:

- Totipotent (or Omnipotent) Cells differentiate in each extraembryonic and embyonic tissue. These cells are derived from embryos at the 4-8 cell stage, after 1-3 days after fertilization.

- Pluripotent Cells are embryonic cells at the blastocyst stage, after 4-14 days after fertilization. These cells are capable of differentiating in tissues of embryonic origin organized in three different germ layers (ectoderm, mesoderm and endoderm), includind germ cells.

- Multipotent Cells have the capacity to multiply and remain in culture, but not the one to be indefinitely renewed. These cells differentiate into different tissues but (except spermatogonial stem cells) cannot differentiate into germ cells. Adult stem cells belong to this category.

- Oligopotent Cells form 2 or more lineages with a specific tissue

- Unipotent Cells present in adult tissues, potentially more limited as well as organ-specific, are able to self-renewal and to differentiate into the cell type of the belonging tissue ensuring its repair and maintenance [39].

Finally there is a classification based on origin that categorizes stem cells in:

A. Embryonic Stem Cells (ESCs) and Embryonic Germ Cells (EGCs) are pluripotent cells, derived from either the inner cell mass of the blastocyst or from primordial germ cells, respectively;

B. Fetal Stem Cells (FSCs) are multipotent cells, derived from fetal blood and bone marrow as well as from other fetal tissues;

C. Adult (or Somatic) Stem Cells (ASCs) are oligopotent cells, derived from adult tissue;

D. Induced Pluripotent Cells (IPSs) are pluripotent cells that are produced from adult somatic cells that are genetically reprogrammede to an “ESC-like state” [39].

2.3.1 CARDIAC STEM CELLS

Recent studies in animal models and humans have shown that both the cardiomyocytes and the vascular cells can regenerate de novo in the adult heart, refuting the paradigm according to which the heart would be a post-mitotic organ composed of terminally differentiated cells that carry their function throughout the entire organism life [40].
The demonstration that humans can divide and amplify small myocytes after an infarction [40, 41] or a pressure overload [43, 44], and the evidence that the mammalian heart has a reserve of stem cells have led to consider the possibility of reconstituting, after a heart attack, the necrotic myocardium and to repair the dilation and thinning of the ventricular wall.
Cardiac Stem Cells (CSCs) identified by the expression of stem cell antigens c-kit (Stem Cell Factor Receptor), MDR-1 (MultiDrug Resistance Protein) and Sca-1 (Stem Cell Antigen) were found in hearts of different mammalian species; these cells have typical properties of stem cells, infact they are multipotent, clonogenic and capable of self-renewal [45].
Cardiac Stem Cells were also obtained from human heart samples by an in vitro selection under stringent conditions that could discourage the expansion of differentiated cell contaminants. In this manner, Cardiosphere Derived Cells (CDCs) and Multipotent Adult Stem Cells (MASCs) were identified in human hearts [46]. These cells are positive for c-kit, also known as CD117, are able to differentiate into the three major heart cell lines (myocytes, smooth muscle, and endothelial cells) and, when injected locally in the infarcted myocardium of immunodeficient mice and immunosuppressed rats, are capable of regenerating damaged tissue, giving rise to new cardiomyocytes and coronary vessels cells [47].
Although it has now been established that there is a population of CSCs in human adult heart, their origin remains still much debated. Some authors argue that resident cardiac stem cells, after being generated during embryonic development, remain in situ in an undifferentiated state, staying mainly in the apex and in the atrium [48, 49]; other studies have recently suggested the epicardium as a possible reserve of multipotent stem cells [50, 51]. In contrast, studies of sex-mismatched cardiac transplants support the hypothesis of an extracardiac origin, suggesting that CSCs would derive from the bone marrow and would migrate into the site of damage after an appropriate stimulation [52, 53].

A recent work by our laboratory has shown that, the transcriptional profile of human CSCs is similar to that of mesothelial cells, supporting the notion that these cells may be derived from the epicardium [54].
A correlation between these two hypothesis has beene found by recent in vitro experimental approaches that have shown as bone-marrow-derived mesenchymal stem cells promote the proliferation and differentiation of CSC residents, through a paracrine mechanism [55, 56].

2.4 CELL SENESCENCE

Cellular senescence can be defined as a stable arrest of the cell cycle coupled to stereotyped phenotypic changes [57].

Mechanisms able to induce senescence can be divided in two principal groups:
1. Intrinsic Mechanisms originating internally to the cell;
2. Extrinsic Mechanisms coming from the outside of the cell.

Intrinsic inducers are either the progressive shortening of telomers with cell divisions (replicative senescence) or the onset of irreparable DNA lesions that induce a persistent DNA Damage Response (DDR), which keeps the cells metabolically active but arrests their growth (telomere-indipendent, premature senescence) [58]. In the latter, intrinsic cell senescence is induced by activated oncogenes and DNA double-strand break-inducing agents. In this regard, 50 oncogenic or mitogenic alterations that are able to induce senescence have been described [59]. Once senescence has been established, senescent cells show remarkable changes in gene expression, including changes in known cell-cycle inhibitors or activators. The cyclin-dependent kinase inhibitor $p16^{INK4A}$ is one of the most important since its expression correlates with the chronological age of essentially all tissues studied, both in mice and humans [60]. In addition to changes in gene expression, senescent cells present also a reorganization of chromatin into discrete foci that are known as senescence-associatedheterochromatin foci (SAHFs). SAHFs are generated in response to activated oncogene in a cell type- and insult-dependent manner and are often associated with the expression of $p16^{INK4A}$ [61].
Extrinsic inducers as Interleukin (IL)-1β, IL-6, IL-8, angiotensin II, Insulin-like Growth Factor-Binding Protein 7 (IGFBP7), Growth-Regulated Oncogene protein Alpha (GROα), Plasminogen Activator Inhibitor 1 (PAI1) and glycation end products have been described to induce senescence in different cell types [58, 62].
Intrinsic and extrinsic inducers are interconnected by the fact that senescence cells exhibit peculiar altered secretome known as Senescence-Associated Secretory Phenotype (SASP) by which they are able to influence their microenvironment, to remodel the extracellular matrix, and to stimulate inflammation creating the conditions to induce cellular senescence in neighboring cells in a paracrine fashion [58].

2.4.1 CONNECTION BETWEEN CELL SENESCENCE AND HEART FAILURE

Growing demonstrations describe a cause-effect correlation among cellular senescence, aging and cardiovascular disease, including heart failure [63]. Histological studies described the association with cardiac aging, senescence and death of CSCs and cardiac myocytes **(Figure XX)** [64, 65]. In line with these statements, other scientists illustrated that, while in acute ischemic heart disease, the pathologic conditions is followed by CSC activation and recruitment toward injured areas, chronic cardiomyopathy is coupled with stem cell senescence apoptosis [49]. In our laboratory, it has been demonstrated that age and pathological conditions are both associated with a reduction in CSC telomerase activity and with an increased accumulation in vitro of cells exhibiting shorter telomeres, telomere-induced dysfunction foci, and expressing $p16^{INK4A}$ and $p21^{CIP}$. Additionally, these cells were characterized by impaired proliferative, migratory and clonogenic capabilities and by an altered gene expression profile, enriched in transcripts of proteins involved in the SASP, such as IL-6 and IGFBP7 [63].

2.5 PROTEOSTASIS AND CARDIOMYOPATHIES

Proteins are involved in almost every biological processes. They are synthesized on ribosomes as linear chain of up to several thousand aminoacids.
To function, proteins must retain conformational flexibility and thus are only thermodynamically stable in their physiological environment. A substantial fraction of all proteins have a three-dimension structure and take folded conformations only after interaction with binding partners [64]. Most of the protein is partially stable in a physiological environment and this leads to the wrong folding and aggregation. In addition, it is estimated that about 30% of eukaryotic proteins is inherently unstructured and contains regions that can only fold in the presence of their ligands. Proteins partially folded or poorly-folded usually expose on their surface hydrophobic amino acid residues and polypeptide regions not-structured, conditions predisposing to an aggregation concentration-dependent. Although the denaturing ports mainly to the formation of amorphous aggregates, it can also lead to the formation of fibrils similar to amyloidosis **(Figure 2.6)**.

Several experimental evidences seem to indicate that alterations of the regulation of normal state of protein folding, the maintenance of the integrity of the proteome and protein homeostasis are the basis for numerous pathological conditions, many of which are related to aging and degenerative diseases.
These alterations of protein, also known as "proteostasis", include diseases caused by loss of function (e.g. cystic fibrosis) and diseases resulting from "functional gain toxic" (Alzheimer's, Parkinson's and Huntington's disease).

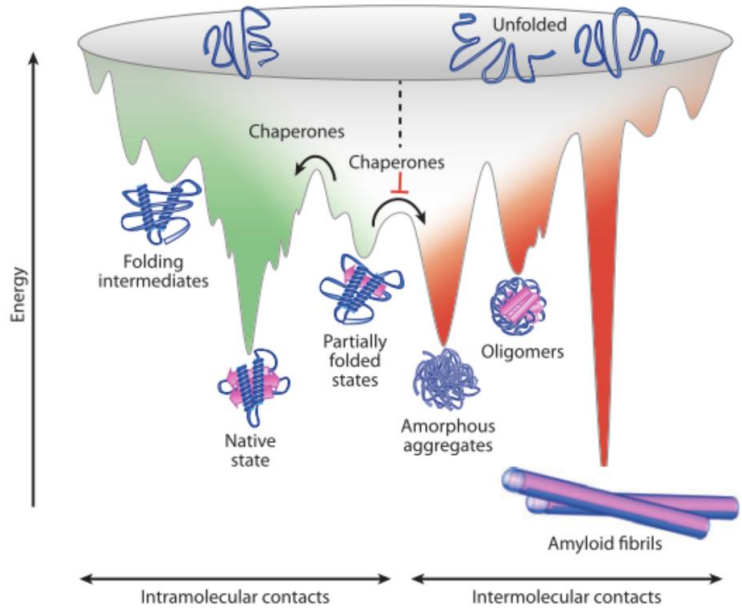


Figure **2.6** Schematic representation of conformational state of proteins related to their energetic state. Intramolecular energetic interactions favorable (green) are stabilized as the protein progresses towards his native state of folding. Proteins trapped in a state of partial folding (relative minima of the free energy) are helped by the chaperones to cross the energy barrier to reach the correct folding. Similarly, intermolecular interactions (red) leading to aggregation are prevented by the action of chaperones. Taken from: Kim Y.E., Hipp M.S., Bracher A., Hayer-Hartl M.,& Hartl F.U. (2013). **Molecular chaperone function in protein folding and proteostasis**. Annual review of biochemistry (Vol.82).

The proteostasis is constituted by a functional network that includes pathways that control the synthesis, folding, trafficking, aggregation, disaggregation, and degradation of proteins. The decrease in the ability of the network to cope with hereditary protein variants prone to aggregate, aging and metabolic stress and/or the environment, it seems to trigger or exacerbate diseases involving loss of proteostasis [65].

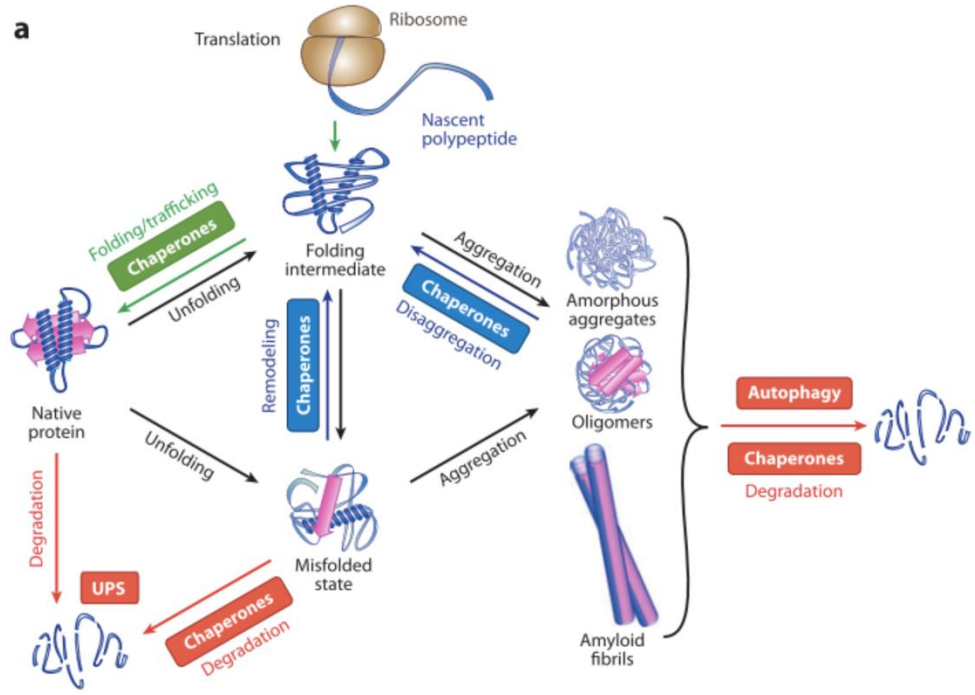


Figure **2.7** Diagram illustrating molecular components mainly involved in the maintenance of proteostasis. Taken from: Kim Y.E., Hipp M.S., Bracher A., Hayer-Hartl M.& Hartl F.U. (2013). **Molecular chaperone functions in protein folding and proteostasis**. Annual review of biochemistry (Vol.82).

The main molecular pathways responsible for maintaining the proteostasis consist of **(Figure 2.7)**:

A) Systems response to stress (such as the system heat shock response cytoplasmic - HSR-, the system response to misfolded protein-UPR- the endoplasmic reticulum and mitochondria).

B) Systems degradation of proteins misfolded (ubiquitin-proteasome system, or UPS)

C) Systems which allow degradation of protein aggregates in the form of small oligomers or fibrils of amyloid (via autophagic-lisosomiale- ALP-).

2.5.1 Heat Shock Response (HSR) and Unfolded Protein Response (UPR)

Proteostasis assists and maintains correct protein folding in cells that face physical and chemical challenges and stresses and is, therefore, crucial to cellular health and survival. It alleviates misfolded protein load by improving protein folding through induction of molecular chaperones, by attenuating protein translation, and by enhancing degradation of terminally misfolded proteins. The Proteostasis network (PN) is regulated by highly conserved genetic and physiologic stress sensors, which include the Heat-Shock Response (HSR) for the cytoplasm and nucleus, the Unfolded Protein Responses (UPRs)

for the endoplasmic reticulum and mitochondria, and other stress signaling pathways that control the expression of molecular chaperones to prevent misfolding and aggregation and allow the clearance of damaged proteins **(Figure 2.8)** [66, 67, 68].

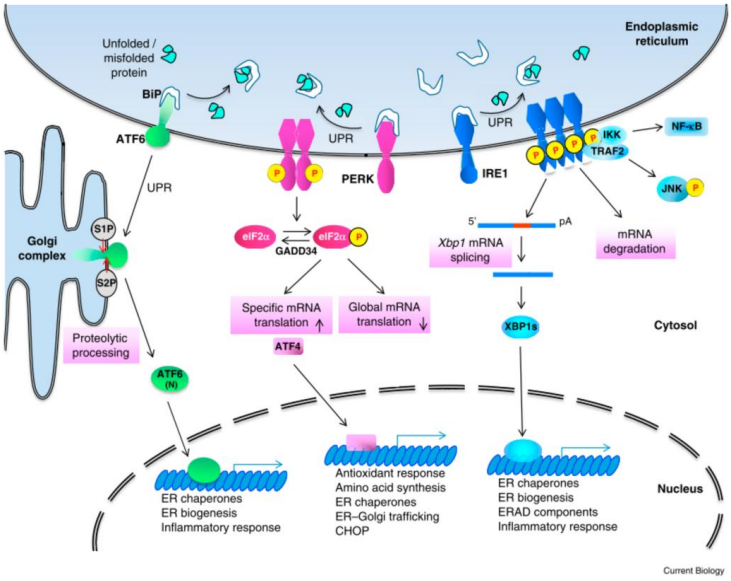


Figure 2.8 Diagram illustrating UPR^{ER} mechanisms in eukaryotes. Taken from: Cao S.S. & Kaufman R.J. (2012). **Unfolded protein response.** Current Biology, 22(16), R622-R626.

The HSR manages denatured proteins in the cytosol [69]. The major player is the transcription factor heat shock factor 1 (HSF1), which is activated during cellular stress and induces the transcription of chaperones and other protective genes. Under normal conditions HSF1 is bound to the cytosolic chaperones heat shock protein (HSP)90 and HSP70 in a monomeric inactive form. When the load of unfolded proteins increases, these chaperones will dissociate from HSF1 and be recruited to unfolded proteins. HSF1 then trimerizes and translocates to the nucleus, where it is post-translationally modified and activates the transcription of HSPs.

The processes of protein folding and maturation are strongly influenced by changes in the homeostasis of the endoplasmic reticulum (ER), such as depletion of Ca^{2+}, increased oxidative stress, hypoxia, energy deprivation, metabolic stimulation, altered glycosylation, activation of inflammation and increased protein synthesis. The accumulation of misfolded proteins in the endoplasmic reticulum triggers a series of events, called UPR, designed to increase the capacity of the ER protein folding, to reduce protein synthesis and stimulate the global degradation of misfolded proteins. The two main streets of this response are initiated by proteins IRE1 and PERK. A series of pathological or environmental conditions may also disrupt the mitochondria.

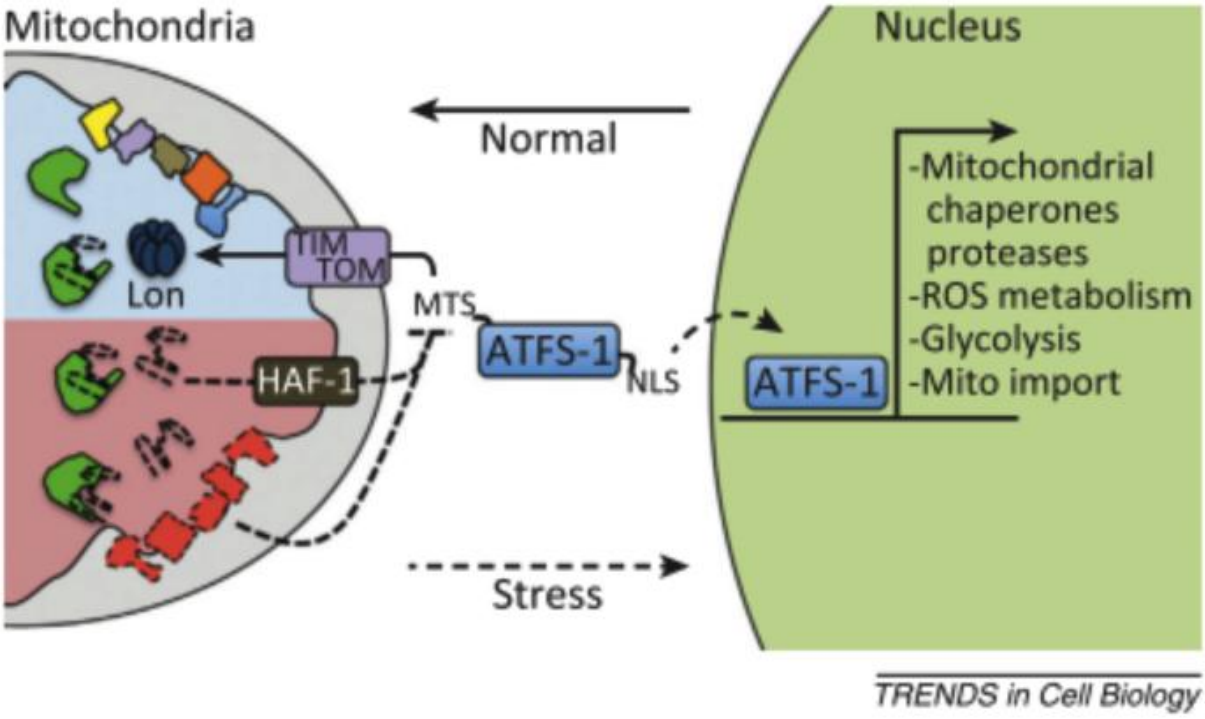


Figure 2.9 Role of the transcription factor ATFS-1 in UPRmt. ATFS-1 is transferred into the mitochondria and degraded by proteases Lon in physiological conditions. In stress conditions, it is accumulated in the nucleus and active its transcriptional program. Taken from: Haynes C.M., Fiorese C.J. & Lin Y.F. (2013). **Evaluating and responding to mitochondrial dysfunction: the mitochondrial unfolded-protein response and beyond.** Trends in Cell Biology, 23(7), 311-318.

Therefore, they have evolved a number of ways mitochondrial-nuclear adapted to indicate the presence of stress or mitochondrial dysfunction and orchestrate the appropriate responses. In particular, the cell seems to monitor the ability of mitochondrial and amount, in the presence of its alterations, activate transcription of a number of target genes (the so-called response UPR^{mt}, **Figure 2.9**) concerted by transcriptional factor ATFS-1. T
The mitochondria can undergo autophagy (in this case, the process is called mitophagy) by a mechanism that involves the protein PINK1.

2.5.2 Ubiquitin- Proteasome System (UPS)

The Ubiquitin-Proteasome System (UPS) participates in the degradation of soluble proteins in cytosol and nucleus. It controls many important biological processes such as cell proliferation, adaptation to stress, cell death and its main function is to prevent the accumulation of damaged, misfolded and changed proteins [70].

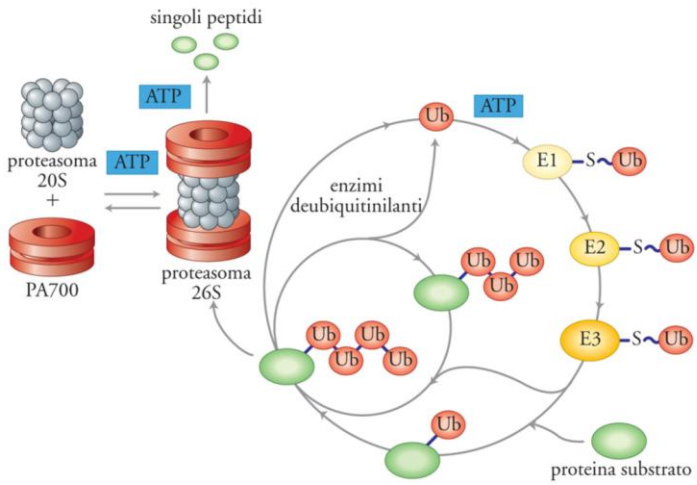


Figure 2.10 Diagram illustrating UPS system. Taken from: Hochstrasser M. (2007). **Proteine, Degradazione delle proteine**. Enciclopedia della Scienza e della Tecnica.

The central core of the UPS is the 20S proteasome, which is present in animals, plants and bacteria. This 700 kDa-multisubunit protease is highly effective in the proteolytic degradation of misfolded or otherwise altered proteins. In eukaryotes, the 20S proteasome binds one or two regulatory 19S complexes to give rise to the 26S proteasome, changing its activity and specificity towards native folded proteins. The function of the 19S subunit is recognized, ubiquitination and open target proteins, then translocate them in the 20S subunit, which possesses proteolytic activity within its central chamber [71].

Generally, UPS-mediated protein degradation consists of two major processes: (1) ubiquitination of the substrate protein molecule and (2) 26S proteasome-mediated degradation of the ubiquitinated protein. In both cases, ubiquitination is achieved by a cascade of enzymatic reactions involving the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3), where the E3 confers substrate specificity and catalyzes the rate-limiting step of ubiquitination. An E3 may be able to ubiquitinate several target proteins while a protein may be targeted for ubiquitination [72].

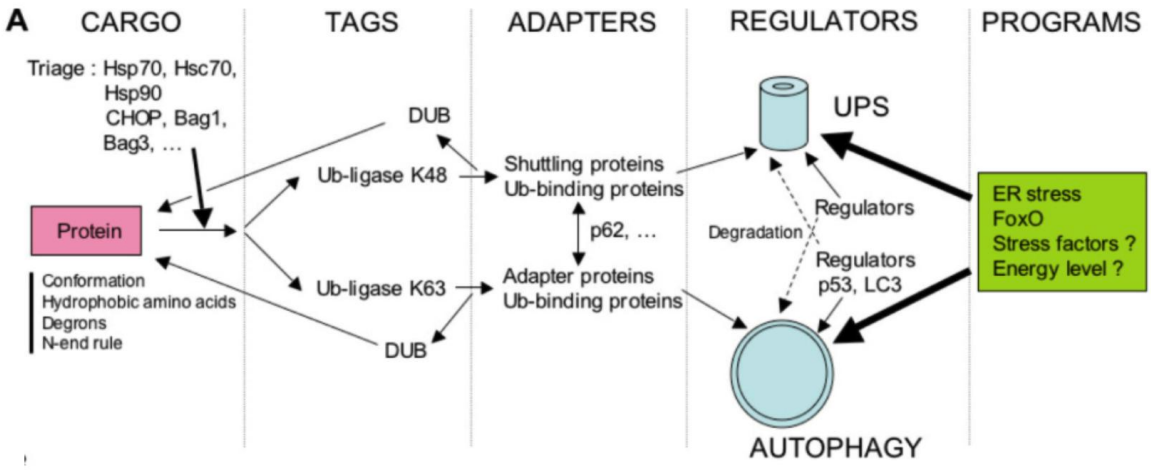


Figure 2.11 CARGO switching between UPS system and ALP system. Taken from: Lilienbaum A. (2013). **Relationship between the proteasomal system and autophagy.** International Journal of Biochemistry and Molecular Biology, 4(1), 1-26.

There is a correlation between UPS and autophagy. Recent developments in the understanding of the mechanisms of regulation of the UPS and the induction of autophagy have clarified the relationship between the two systems. The trade-union between the two systems is represented by proteins and p62SQSTM1 NBR1. They act as a receptor for the selection of ubiquitinated substrates to be sent to degradation by autophagy **(Figure 2.11)**.

2.5.3 Autophagy

Autophagy usually occurs at low basal levels to maintain the cellular homeostasis, and could be stimulated in response to various cellular stresses, such as nutrient limitation, oxidative stress and ER stress.

The major pathways for degradation of cellular constituents are autophagy and cytosolic turnover by the proteasome. These degradative pathways are particularly important during development and under certain environmental stress conditions.

In eukaryotic cells, the lysosome or vacuole is a major degradative organelle. This compartment contains a range of hydrolases that are able to degrade essentially any subcellular constituent (proteins, lipids, nucleic acids, and carbohydrates). Regulated turnover of organelles is confined to the lysosome. Cytoplasmic components are degraded within the lysosome by microautophagy, chaperone-mediated autophagy, and macroautophagy **(Figure 2.12)** [73, 74].

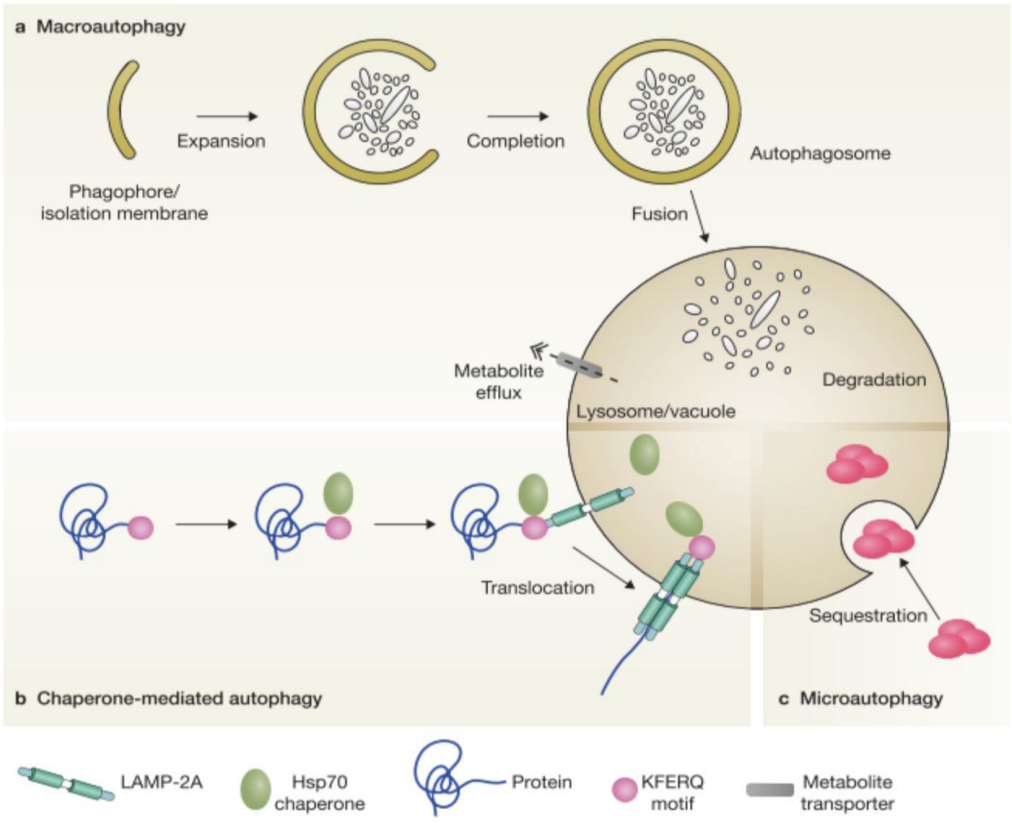


Figure 2.12 Diagram Illustrating three principal types of autophagy. Taken from: Boya P., Reggiori F. & Codogno P. (2013). **Emerging regulation and functions of autophagy.** Nature Cell Biology, 15(7), 713-20.

The ***micro-autophagy*** digests cytosolic components that are taken directly from the lysosome by invagination of their membrane.

In ***chaperone-mediated autophagy (CMA)***, cytosolic proteins bearing a targeting motif biochemically related to the pentapeptide KFERQ [75] are recognized by the chaperone hsc70 that delivers them one-by-one to lysosomes for degradation [76]. Chaperone-mediated autophagy is distinct from other types of autophagy in that it does not require sequestration of the proteins to be degraded (cargo) into any type of vesicles for lysosomal delivery. Instead, a transmembrane protein, the lysosome-associated membrane protein type 2A (LAMP-2A) [77], serves as both the CMA lysosomal receptor to which substrate proteins bind, and the main component of the complex that facilitates their translocation into the lysosomal lumen. Chaperone-mediated autophagy function declines with age and is also compromised in diseases such as neurodegenerative conditions, metabolic disorders, and cancer.

Macroautophagy, defined Autophagy, is a morphologically and genetically defined process which is coordinated by a series of autophagy-related (ATG) genes. Macroautophagy is characterized by the formation of double-membraned vesicles termed autophagosomes **(Figure 2.13)** [78].

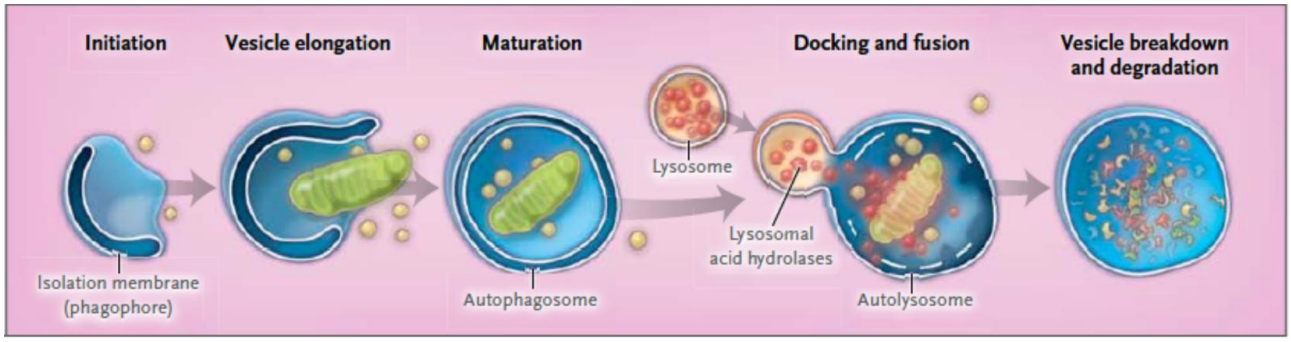


Figure 2.13 Diagram illustrating autophagic process phases. Taken from: Choi A.M.K., Ryter S.W. And Levine B. **Autophagy in Human Health and Disease.** N. Engl J Med 2013;368:651-62.

These vesicles serve to carry cargoes destined for digestion at the lysosome **(Figure 2.14)**. Upon initiation, membranes, termed phagophores, are formed from various sources within the cell [79]. As these membranes grow, a cleaved form of the protein MAP1LC3B (termed LC3-I), which is expressed diffusely in the cytoplasm, is conjugated to the lipid phosphatidylethanolamine [80]. The protein, after this process, is termed LC3-II, and through its lipid conjugate integrates into the phagophore membrane.Once the autophagosome is formed, it can undergo fusion events with other vesicles including endosomes and multivesicular bodies, but ultimately autophagosomes fuse with lysosomes to form autolysosomes. It is in autolysosomes that the cargo of the autophagosome is digested by acid hydrolases provided by the lysosome, and the breakdown products are then transferred back into the cytoplasm where they can either be recycled or further catabolized to fuel metabolism.

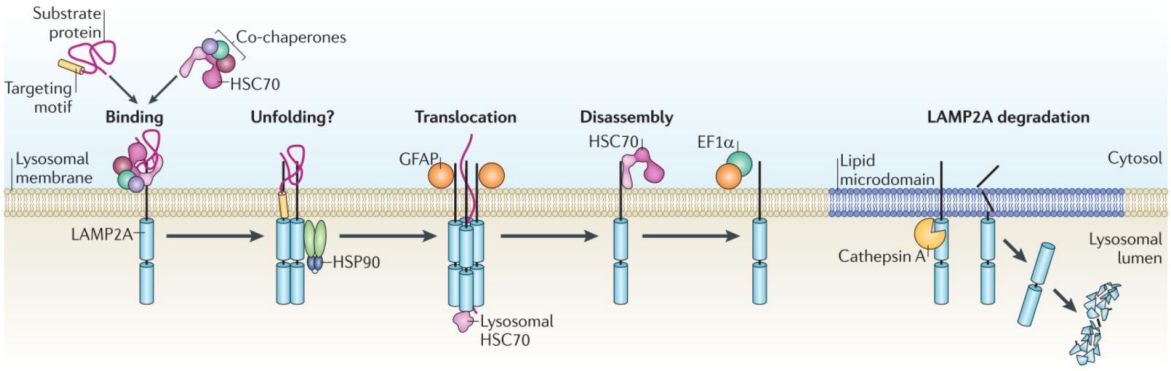


Figure 2.14 Diagram representing molecular mechanisms of CMA working. Taken from: Cuervo A.M. (2011): **Chaperone-mediated autophagy: Dice's "wild" idea about lysosomal selectivity.** Nature Reviews. Molecular Cell Biology, 12(8), 535-541.

The molecular mechanisms acts to trigger and regulate autophagy have been clarified in detail and involve at least 5 molecular components (**Figure 2.15**), including:
1) the complex Atg1 / unc-51-like kinase (ULK),
2) the complex Beclin 1 / class III phosphatidylinositol 3-kinase (PI3K),
3) transmembrane proteins Atg9 and vacuole membrane protein 1 (VMP1),
4) two systems of ubiquitin-like protein conjugation (Atg12 and Atg8 / LC3),
5) proteins that mediate the fusion between autophagosomes and lysosomes.

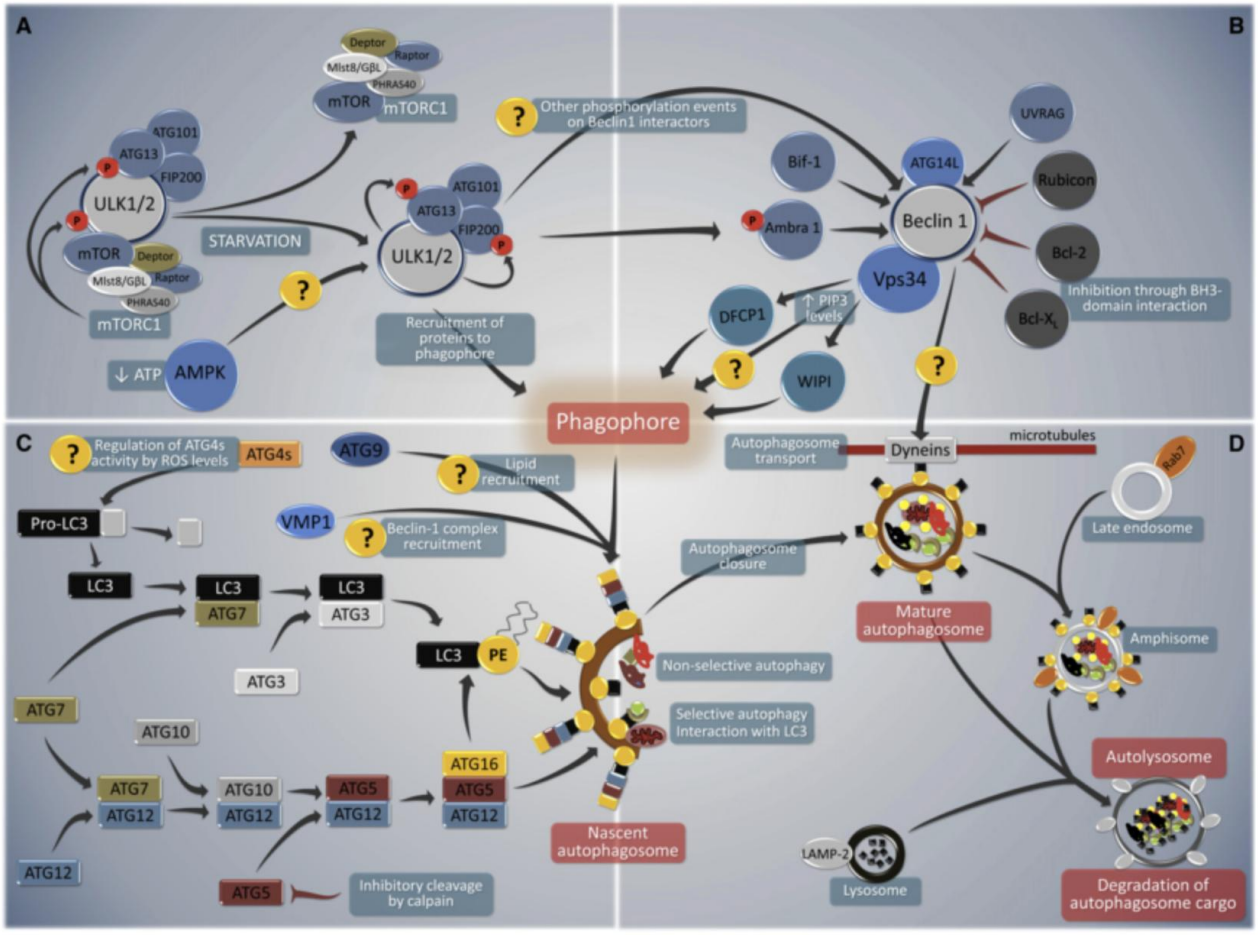


Figure 2.15 Molecular mechanisms of autophagy regulation. Taken from: Kroemer G., Marifio G. & Levine B. (2010). **Autophagy and the Integrated Stress Response**. Molecular Cell, 40(2); 280-293.

The deprivation of nutrients (via the signaling pathways of AMPK, mTOR and Sirtuin) because different types of stress (UPR^{ER}, hypoxia anoxia, oxidative stress, mitochondrial dysfunction and activation of p53) are able to activate autophagy. Important is the relationship between autophagy and apoptosis [81].

2.5.4 Lysosomes

Discovered by Christian de Duve over 50 years ago [82], the lysosome is a cytoplasmic cellular organelle. Present in all nucleated eukaryotic cells, the lysosome is delimited by a single-layer lipid membrane and has an acidic internal pH (~ 5) that is maintained by an ATP-dependent proton pump. The primary cellular function of the lysosome is the degradation and recycling of macromolecules obtained by endocytosis, autophagy and other cellular trafficking pathways. Several classes of macromolecules are hydrolyzed including proteins, polysaccharides, lipids and nucleic acids and this is achieved by the concerted action of numerous soluble catabolic enzymes within the lumen of the lysosome, collectively termed acid hydrolases. Acid hydrolases have evolved to function in the low pH of this organelle and possess a wide variety of enzymatic properties.

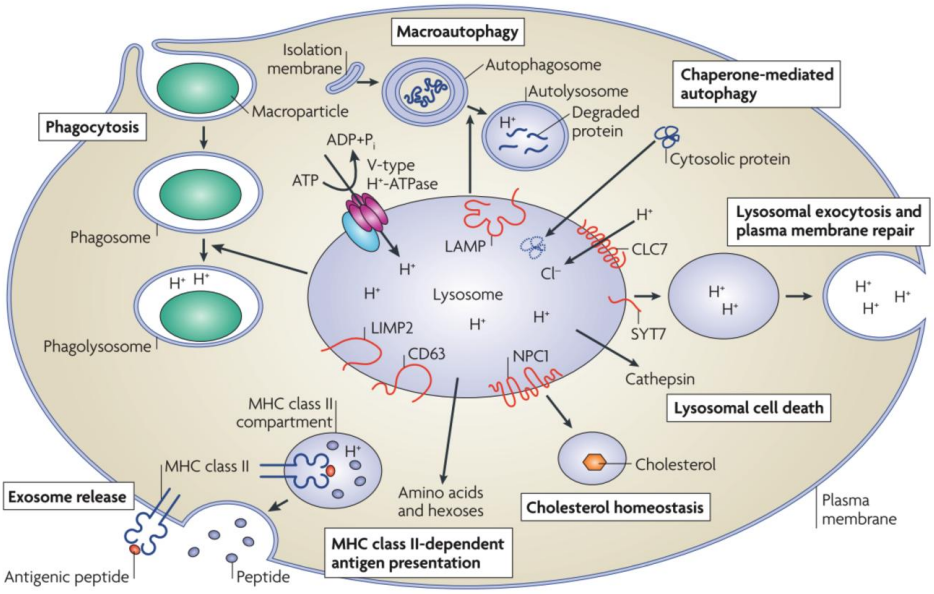


Figure 2.16: Convergence of Different digestive pathway to lysosome. Taken fom: Saftig P. & Klumpermann J. (2009). **Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function.** Nature Reviews Molecular Cell Biology, 10(9), 623-635.

The lysosomal integral membrane proteins (called LMP) have different functions, such as acidification of lysosomal lumen, the import of proteins from the cytosol, the movement lysosomal membrane fusion and the transport of degradation products in the cytoplasm [83]. In addition to the role in the regulation of the final stages of catabolic processes and in the processing of pre-proteins, the lysosomes are also involved in the processing of antigens in the extracellular matrix degradation, in the repair of plasmatic membranes and in the initiation of apoptosis, a lysosomal dysfunction has a profound impact on cellular

homeostasis and is associated with infectious diseases, neurodegenerative and aging (**Figure 2.16**).

Different degradation pathways converge to the lysosome, including endocytosis, phagocytosis and autophagy. The first two tracks degrade components of the extracellular environment (heterophagia) while autophagy macro, micro and chaperone-mediated (CMA) mainly degrade the intracellular components [84]. The lysosomes are organelles the main catabolic and degrade a plethora of compounds, including surface receptors, macromolecules, organelles and pathogens, and some short-lived proteins previously degraded by the proteasome system. In addition, the lysosomes digest also intracellular organelles that reach the lysosomal lumen via macroautophagy, as well as the mitochondria, peroxisomes, and the endoplasmic reticulum.
Given their high levels of hydrolytic enzymes, lysosomes are potentially harmful to the cell. Because of the strong against the lysosomal membrane, it occurs the release of its contents into the cytoplasm, inducing the indiscriminate degradation of cellular components. A massive lysosomal rupture can induce cytosolic acidification, which in turn can induce cell death by necrosis [85].
Lysosomal biogenesis requires the integration of the endocytic and biosynthetic pathways of the cell. The targeting of newly synthesized proteins to lysosomes can be direct from the *trans-Golgi network* or indirectly through the membrane transport palsmatica followed by endocytosis. Less clear is the addressing mode of the LMP to lysosomes. Recent studies suggest that several genes encoding lysosomal proteins exhibit a behavior transcriptional coordinated, regulated by the transcription factor EB (TFEB) [86].

2.5.5 GALECTINS AND LYSOSOMES

Galectins are soluble carbohydrate-binding lectins defined by their ability to bind β-galactoside sugars with one or two conserved carbohydrate-recognition domains. To date, ten human galectins with different expression patterns and sugar binding affinites have been identified, and highly conserved members of this family are present in organisms from roundworms to mammals.
Galectins are present in the cytosol and nucleus as well as in the extracellular space. The binding of extracellular galectins to cell surface glycans can modulate cellular behavior by regulating transmembrane signaling as well as cell-cell and cell-matrix interactions.
Recent studies have demonstrated that in the absence of bacteria or viruses, some galectins can translocate to damaged endo-lysosomes before their autophagy-mediated removal. Prompted by these data, the sensitivity and specificity of galectin punctae formation is used as a novel assay for Lysosomal membrane permeabilization.
Galectin1 and Galectin3 are best suited for LMP detection. In our study we evalueted the Galectin3 localization for LMP detection. Punctae of galectins absent in healthy cells and their formation takes place regardless of how the lysosome has been damaged: this suggests that the translocation of them to the lysosome is a general consequence of the rupture of the lysosomal membrane. The punctae of galectin persist for several hours regardless of the fate of the cell.

2.6 PROTEOTOXICITY AND CARDIOMYOPATHIES

Proteotoxicity is linked to protein homeostasis. The balance of protein synthesis and degradation in each cell is highly regulated and occurs in a specific manner to maintain cellular homeostasis. However, under the circumstances of cardiac remodeling during heart disease this balance can be altered leading to accumulation of potentially toxic proteins. To ensure that these misfolded or aberrant proteins are either repaired or removed, a set of molecular mechanisms works in collaboration or separately as a quality control of the cell. This quality control consists of molecular chaperones and co-chaperones, the autophagy-lysosomal pathway (ALP), and the ubiquitin-proteasome system (UPS). UPS alteration is rapidly gaining recognition as a major player in the pathogenesis of several cardiac disorders, including inherited cardiomyopathies (**Figure 2.17**).

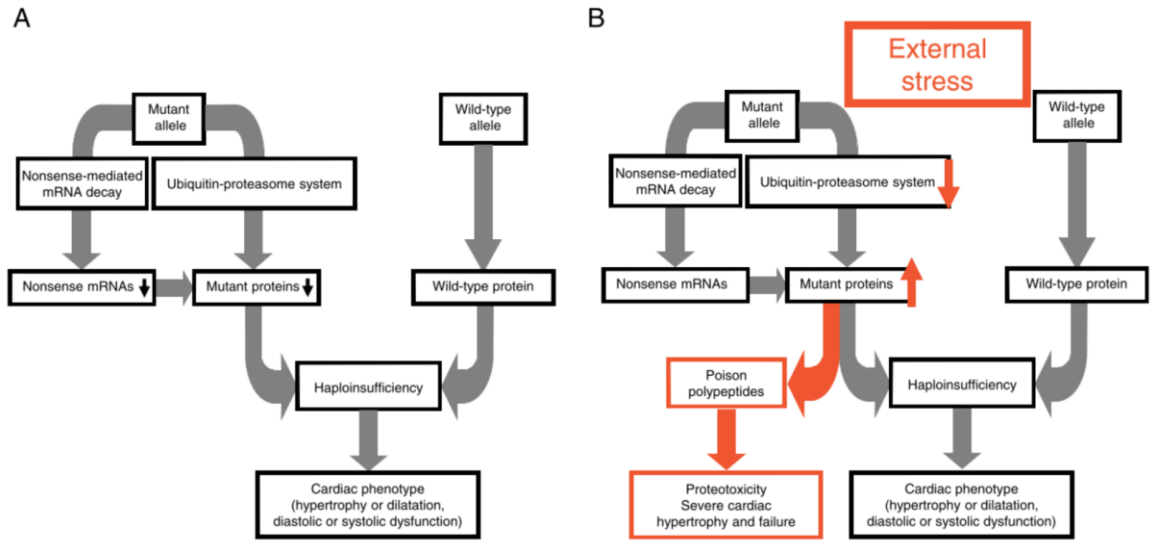


Figure 2.17: Mechanisms which compensate the presence of a mutated allele in a patient affected by cardiomyopathy (a) and consequences of compensation mechanisms loss (B). Taken from: Sclossarek S., Frey N. & Carrier L. (2014). **Ubiquitin-proteasome system and hereditary cardiomyopathies.** Journal of Molecular and Cellular Cardiology, 71, 25-31.

The UPS controls many fundamental biological processes such as cell proliferation, adaptation to stress and cell death, and its major function is to prevent accumulation of damaged, misfolded and mutant proteins [87]. Degradation of proteins by the UPS is an ATP-dependent multistage process that requires first ubiquitination of the target protein prior its degradation by the 26S proteasome [88].

Most cases of heart failure with hypertrophic, dilated or ischemic cardiomyopathies exhibit accumulation of ubiquitinated proteins [88], abnormal protein aggregation such as pre-amyloid oligomer formation [90], and altered proteasomal activities [91].

Mutant misfolded proteins are recognized by chaperones (such as Hsp70 and Hsp90) and co-chaperones (such as CHIP, and bag1 BAG3) who will handle their correct folding or their degradation by the UPS system and / or autophagic-lysosomal. Similarly, in mice Knock-In for titin gene mutations present in families affected by DCM, the mRNA of the mutated allele it is degraded so that in compensation mechanisms prevails protein WT and mice did not show an obvious phenotype [92].

Different mechanisms may lead, separately or in combination, to defects and loss of UPS mechanisms compensation.

First, the continued degradation of mutant proteins by UPS can lead to saturation of this system as occurs in desmin-related cardiomyopathy due to mutation of the gene coding for the small Heat Shock Protein *CryAB* [93].

Second, the combination of an external stress and a UPS system overload can cause unbalancing this system. This happens, for example, in some mouse models of HCM and DCM, where the UPS system, which degrades continuously mutated proteins of animals bearing mutations in *Lmna, Mybc3 or TTN*, is saturated due to adrenergic stress or due to aging [91]. This could be due to the redirection intracellular proteasome under stress to granules of intracytoplasmic inclusion [93].
Third, the misfolded proteins that are beyond the control of the chaperones and UPS, tend to form the aggregated, that are potentially toxic to the cell. According to this statement, it has been shown to be present a lot of protein aggregates seized in a specialized structure called aggresoma, as intracellular amyloid in cardiac myocytes of animal models and patients with HCM and DCM [90]. In addition, protein aggregates impair the function of the proteasome in cardiac myocytes [93], giving rise to a vicious circle.
Fourth, an increase in oxidative stress involves the formation of protein aggregates. In the case of 'aging, this could be due to increased production of free radicals by mitochondria dysfunctional [92]. Oxidative stress induces oxidation and protein aggregation resulting in binding of oxidized proteins to the 20S subunit of the proteasome and irreversible inhibition of the activity of the latter [93]. This mechanism could trigger a vicious circle leading to accumulation of oxidized proteins, that would normally be degraded by the proteasome system.
Fifth, an assemblage altered proteasome or a change in the distribution of subpopulations proteosomics [93] could lead to compromise of the UPS. A recent study showed a reduced anchoring subunit 19S to 20S in patients with terminal heart failure [93]. This may affect the ability of proteasome degradation and therefore explain the decrease in activity of the proteasome found in this group of patients [66]. most recently, the regulation of the UPS system involves post-translational modifications of the subunits of the proteasome, such as phosphorylation, acetylation or oxidation.
it is possible that the decreased PKA-mediated phosphorylation of components of the proteasome contributes to dysfunction of the UPS in cardiomyopathies.

2.6.1 Potential Consequences of UPS Impairment

The dysfunction of the UPS system profoundly alters the normal physiology of cardiac myocytes, acting at different levels (**Figure 2.18**).
UPS impairment could have several consequences in cardiac myocytes. A number of key proteins involved in cardiac hypertrophy and apoptosis pathways are either targets or components of the UPS. For instance, several signaling proteins, such as β-catenin and calcineurin, which mediate cardiac growth are normally degraded by the UPS.

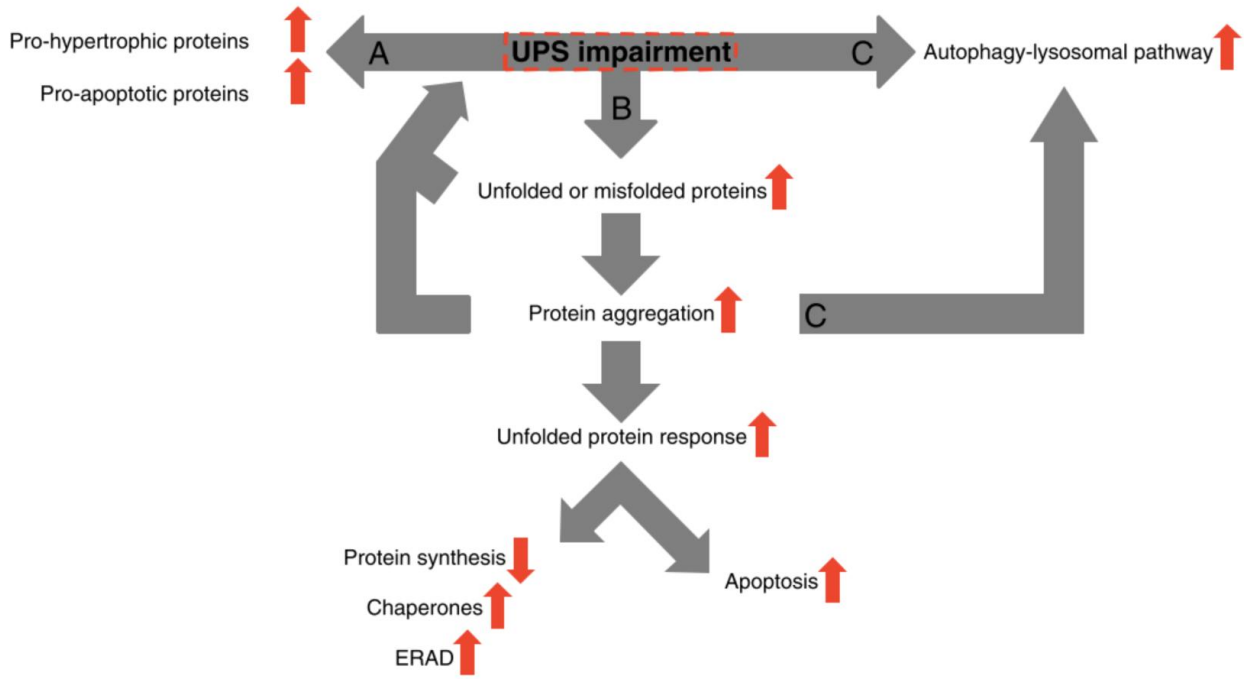


Figure 2.18: Possible consequences of UPS and proteostasis dysfunction in cardiomyocytes. Taken from: Sclossarek S., Frey N. & Carrier L. (2014). **Ubiquitin-proteasome system and hereditary cardiomyopathies.** Journal of Molecular and Cellular Cardiology, 71, 25-31.

UPS impairment could lead to different phenomenons as:

- Accumulation of proteins involved in hypertrophic signaling, as calcineurin, or apoptotic pathway, as p53, which are normally degraded by the UPS.

- Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER), which leads to protein aggregation. This ER stress activates the unfolded protein response, characterized by attenuation of protein synthesis, upregulation of chaperones genes (e.g. GRP78) and ERAD (ER-associated protein degradation). Prolonged stress will lead to apoptosis. Accumulation and/or aggregation of misfolded proteins could itself force UPS impairment.

- Activation of the autophagy-lysosomal pathway directly or indirectly (by protein aggregation) in order to contribute to protein degradation.

UPS impairment could also lead to accumulation of unfolded or misfolded proteins and aggregation of proteins. This might result in ER stress, leading to an adaptive response, which is known as the unfolded protein response (UPR) resulting in attenuation of protein synthesis, transcriptional activation of the genes of chaperone proteins and activation of ER-associated protein degradation (ERAD) [93]. If the answer UPR was prolonged in time, it would trigger a signaling pathway that leads UPR-mediated apoptosis. This hypothesis is supported by experimental data obtained in response to proteasome inhibition in cardiac myocytes, which has the effect death cardiomiocitaria activated by CHOP. Finally, as noted previously, the accumulation and / or aggregation of misfolded proteins have deleterious effect on the UPS system and can trigger a vicious cycle.

Although UPS is usually the main system of protein degradation, the ALP system is mainly responsible for the degradation of proteins with long half-life of protein aggregates and organelles [92].

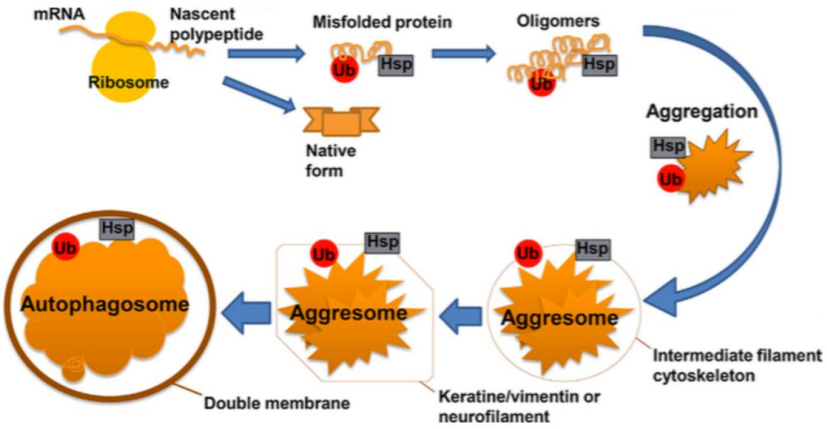


Figure 2.19: Aggregasome formation and ALP pathway stimulation from the latter. Taken from: Hyttinen J.M.T., Amadio M., ViriJ., Pascale A., Salminen A. & Kaarniranta K. (2014). **Clearance of misfolded and aggregated proteins by aggrephagy and implication diseases**. Ageing Research Reviews, 18C, 16-28.

Several lines of evidence indicate that, in the case of failure of the systems mediated by chaperones and protein folding of the UPS system, is activated a specialized form of autophagy deputed to removal of the aggregated protein ubiquitinilated (**Figure 2.19**). In this pathway, misfolded proteins form aggregates of successively larger sizes that take the name of aggresomes, which are sent to degradation by the system ALP.
On the basis of this evidence, it can be concluded that UPS impairment contribute to the pathogenesis of cardiomyopathies.

2.7 AUTOPHAGY AND INFLAMMATION

To respond adequately to infectious agents, the organism must activate both the innate response both the adaptive immune system, including autophagy-dependent functions. In addition, they must be triggered specific responses aimed at limiting the damage resulting immune activation and inflammation. A particularly attractive field of research is the study of the role played by the autophagic proteins in maintaining this delicate balance.
It was shown that autophagy can be modulated by numerous molecules involved in regulating the immune system, such as different families of receptors involved in the recognition of pathogens and molecular profiles associated with the damage -DAMP- (as Toll Like Receptors, NOD-Like Receptors, PKR protein binding RNA-double-stranded), receptors for pathogens (as CD46), INF-γ, JKK, CD40, TNFα, IKK and NF-*k*b. In addition, other cytokines as CLCF1, LIF, IGF1, FGF2, SDF1 and cytokines activating STAT3, are able to inhibit autophagy.
Autophagy is involved in the development and maintenance of adaptive immunity (**Figure 2.20**) including antigen presentation, while autophagy system proteins act as the activators both inactivating of the innate immune signaling. In particular, it appears that the proteins of the autophagic pathway are able to activate the production of interferons by plasmacytoid dendritic cells presenting viral nucleic acids to the endosomal TLR. On the contrary, the autophagic proteins negatively regulate the production of interferons by various mechanisms (for example, removing the dysfunctional mitochondria and adjusting the activation of RIG-I Like receptors).
Important, for the study of this thesis, is the effect exerted by the autophagic pathway on the inflammation control (specifically through the regulation of transcriptional responses pro-inflammatory). In particular, the increased levels of the protein p62, which accumulates in cells with defects of ALP system, determines the activation of the transcription factor pro-inflammatory NF-*k*B through a mechanism that involves the oligomerization of TRAF6 [94].

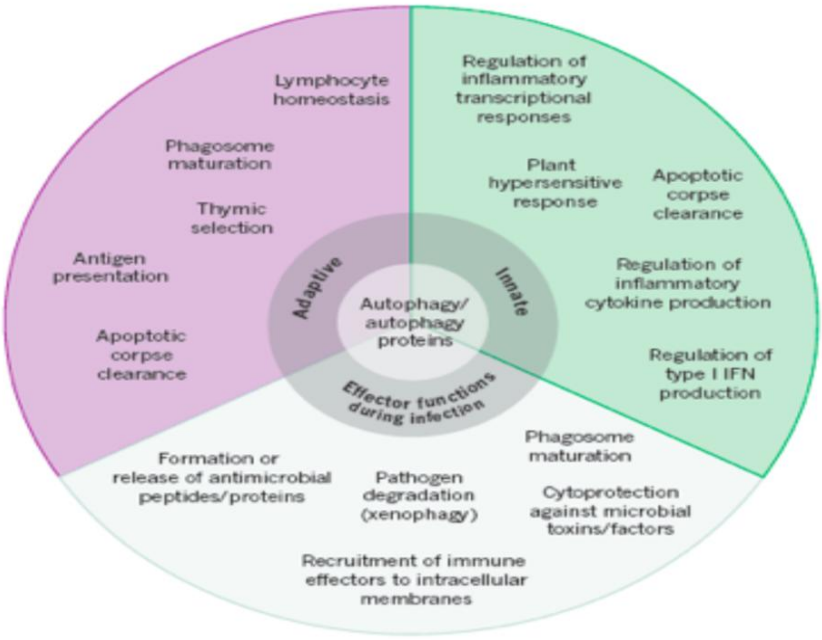


Figure 2.20 Functions of autophagy pathway a/o proteins in immunity. Taken from: Levine B., Mizushima N. & Virgin H.W. **Autophagy in immunity and inflammation.** Nature January 2011, Vol.469.

Another important effect of autophagic protein on inflammatory signal occurs at the level of the inflammasome. This complex, containing receptors NOD, the adapter protein ASC and Caspase 1, is triggered by infection or other stress and promotes cell maturation and secretion of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and IL-18 [93].
The mechanism by which the autophagic proteins negatively regulate the activation of the inflammasome has not yet been fully explained.
Although it is not excluded the possibility of direct interactions between autophagic proteins and some components of the inflammasome, it is believed that the activity of the inflammasome is indirectly inhibited, by preventing the accumulation of ROS implemented through autophagy of dysfunctional mitochondria, or by autophagic degradation of signals that can trigger the inflammasome [94].
To regulating the inflammatory signaling, the autophagic view could prevent inflammation through the clearance of apoptotic bodies, before they proceed toward the secondary necrosis issuing danger signals (DAMPs) capable of activating the inflammation.

2.7.1 Autophagy and Cardiovascular Pathologies

Autophagy alterations have been associated with cardiovascular diseases such as heart disease, cardiac hypertrophy, coronary artery disease, heart failure and stroke [93]. This biological process plays an important role in the physiology of the cardiovascular system, but it is not still unclear if this phenomenon can be considered positive or negative. It has been recently observed that autophagy plays a dual effect, protective under some conditions and negative in the other (**Figure 2.21**).

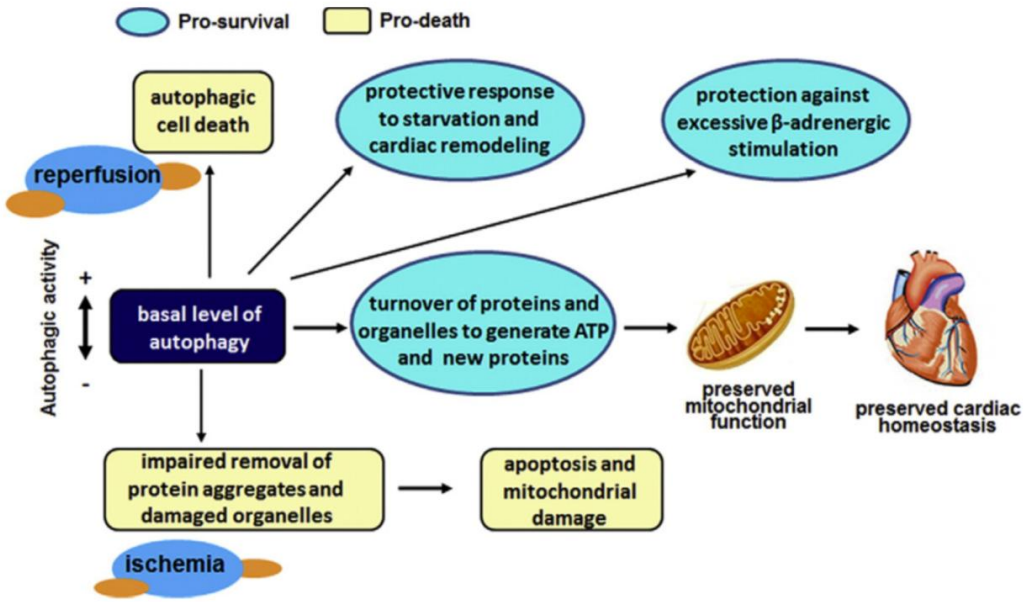


Figure 2.21: Dual role played by autophagy in survival and death of cardiac cells. Taken from: Ren J. & Taegtmeyer H. (2015). **Too Much or Not Enough of a Good Thing- The Janus Faces of Autophagy in Cardiac Fuel and Protein Homeostasis**. Journal of Molecular and Cellular Cardiology. 84, 223-226.

Autophagy appears to play a protective role on cells exposed to different stressors.It was observed that the autophagic flux decreases with advancing age (event that is associated with accumulation of damaged proteins and organelles), whereas stimulation of autophagy is able to slow the aging process. In the presence of ischemic damage, autophagy promotes the removal of damaged mitochondria and at the same time allows the cell to recycle aminoacids and fatty acids for ATP generation. In this context, autophagy is helpful

while, during reperfusion, autophagy is overly stimulated, leading cellular death. This latest concept is much debated but as the accumulation of autophagosomes observed during ischemia / reperfusion injury could be due to a defective lysosomal clearance. In particular, some authors have observed a reduction in the levels of the lysosomal protein LAMP-2.

The correct functionality of the ALP system flow is underlined by the causal role exercised by mutations in the LAMP2 gene in the pathogenesis of Danon disease which is an hypertrophic cardiomyopathy. In this disease, cardiomyocytes show evidence of mitochondrial dysfunction and accumulation of autophagosomes [94]. Similarly, defects autophagic flux were also demonstrated in some primitive familial cardiomyopathies, such as those secondary to lamin A / C mutation or dystrophinopathies.

3 - AIM

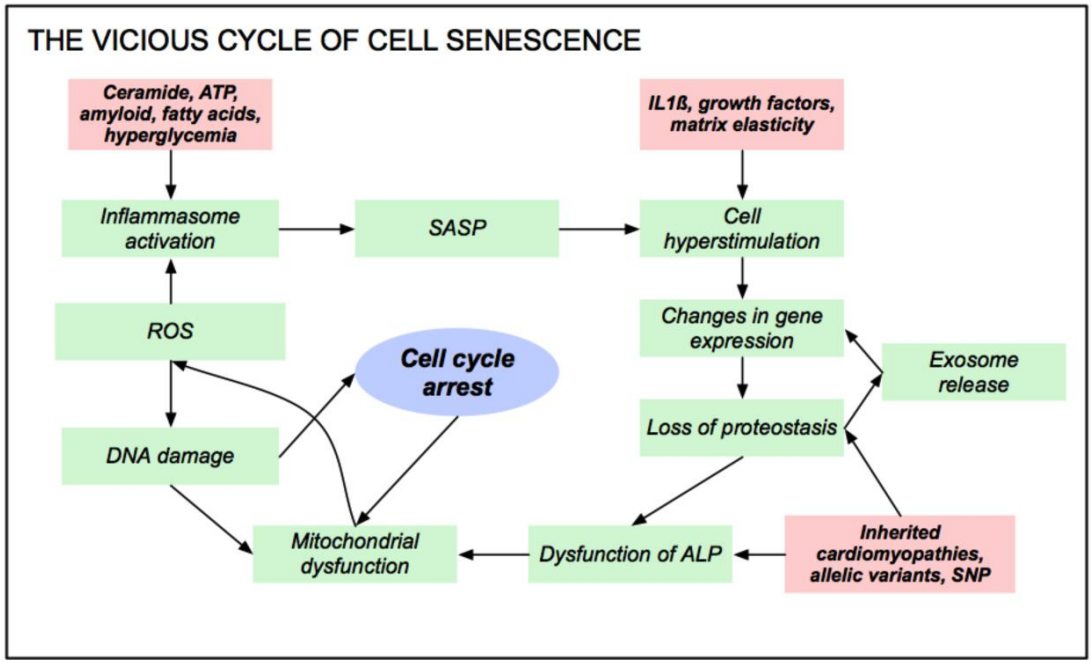


Figure 3.1 Schematic representation of vicious cycle cell senescence. Various environmental stress and conditions predisposing to genetic basis activate a series of linked events which alter myocardiac physiology and cause inflammation promoting heart failure.

Against this background, we hypothesized that, in patients affected by DCM, build a vicious circle (Figure 3.1), characterized by the accumulation of dysfunctional mitochondria, production of reactive oxygen species (ROS), on DNA damage resulting in cell senescence, inflammasome and transcription factors (such as NF-kB) activation, which have a crucial role in the inflammatory response which results in the production of proinflammatory cytokines that can stimulate myocyte hypertrophy, thereby worsening the proteostasi.
Specific objectives of this study were:
1) Mitochondrial dysfunction and production of free radicals (in particular products of lipid peroxidation such as the 4-OHnonenato)
2) Activation of the response to injury in the DNA double helix
3) Activating dell'inflammasoma
4) Possible activation of the transcription factor NF-kB
5) Myocyte hypertrophy

4- RESULTS

The research project carried out in this thesis was conducted at the Institute of Pathology, University of Udine and studies the pathophysiological mechanisms that characterize the evolution of idiopathic dilated cardiomyopathy (iDCM) to heart failure.
We focused our study on left ventricles of 48 patients with iDCM transplanted at the Cardiac Surgery of the University Hospital of Udine (directed by Prof. Ugolino Livi). These samples were compared with samples of myocardium obtained from the front wall of the left ventricle of subjects died from no cardiovascular causes.
Demographic, anatomic and pathologic characteristics of these sample are shown in **Table 4.1**.
The study, in accordance with the Declaration of Helsinki, was approved by Ethics Committee of Udine (2 August 2011, reference number 47831) and written informed consent was obtained from each patient.

	EXPLANTED HEART	**CONTROLS**	**p**
Sample number	48	18	
Sex (M/F)	79,2% / 20,8%	37,5% /62,5%	N.S.
Age	52,5 ± 13,5	40,7 ± 12,4	0,002
Time elapsed between death and picking (hours)	N.A.	14,5 ± 6,1	-
Heart weight	498 ±161	334 ± 109	<0,001
Left Ventricle internal diameter longitudinal (cm)	9,4 ± 1,7	7,2 ± 0,9	<0,001
External transverse diameter (cm)	13,0 ± 1,6	10,5 ± 1,9	<0,001
Genetic and familiar forms	6	N.A.	-
Patients enrolled in the register of cardiomyopathies of Trieste	15	N.A.	-

Table 4.1 Demographic and pathologic characteristics of patients enrolled in the study

As described in the literature, patients affected by iDCM were relatively young (average age of 52 years) and predominantly male. Moreover 6 patients presented familiar dystrophinopathies or a pathological mutation.
The weight of the explanted hearts and inner longitudinal and transverse exterior diameters of the left ventricle were significantly increased compared to controls.

4.1 Ventricular Fibrosis

As discussed in the introduction, a fundamental feature of idiopathic dilated cardiomyopathy is the presence of myocardial fibrosis. To evaluate the accumulation of connective tissue, samples obtained from patients and controls were stained by Gomori trichrome and calculated by morphometric analysis.

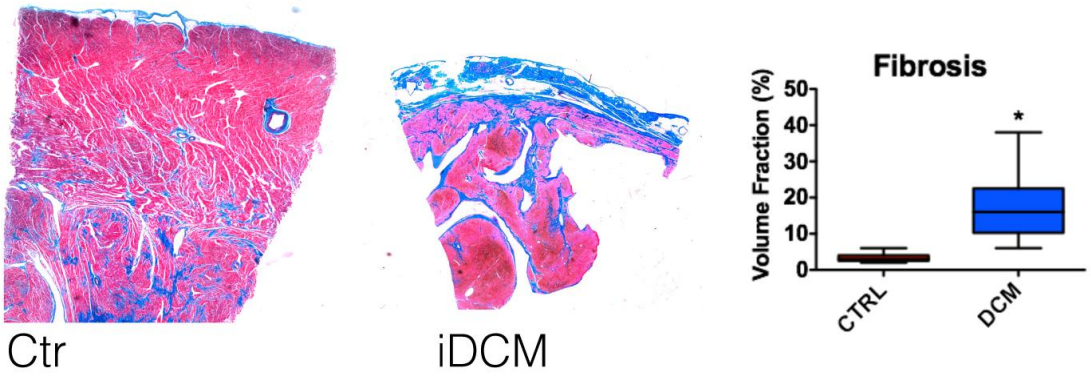


Figure 4.1: Typical examples of hearts obtained by controls and patients affected by iDCM stained by Gomori trichromes. Histograms on the right shows the results of quantification of the volume fraction occupied by myocardial fibrosis. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars).

Hearts explanted by patients affected by idiopathic dilated cardiomyopathy were compared with control hearts and we confirmed that the heart patients are characterized by a significant increase in the volume fraction occupied by myocardial fibrosis, as shown in **Figure 4.1**.

4.2 Loss of Proteostasis

We stained sample obtained from patients and controls with Congo Red alkaline to test proteostasis loss and accumulation of protein aggregates in patients affected by iDCM.
Congo Red is a reagent used to evaluate the deposition of amyloid and has a typical spectrum of excitation / emission (about 488/614 nm, respectively) in the fluorescence

quantification. Amyloid quantification in fluorescence is more sensitive than the classical evaluation optical microscopy in polarized light [95].

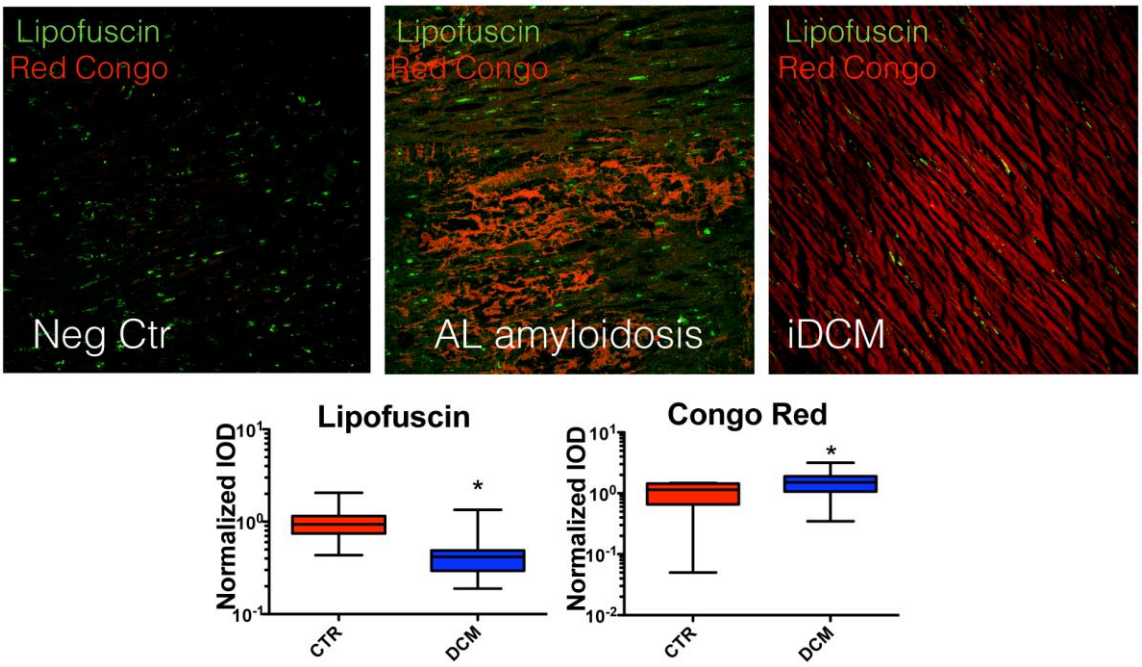


Figure 4.2: Confocal images of myocardial samples obtained by controls, patients affected by amyloidosis AL (positive control) and patients affected by iDCM. Histograms below show the data obtained by quantification of fluorescence. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

The samples taken from patients affected by iDCM are characterized, compared to those obtained from controls, by significantly higher levels of amyloid localized at the intracellular level, as shown in **Figure 4.2**. The presence of lipofuscin, determined by staining with Sudan Black, instead is significantly reduced in patients suffering from DCM.

4.3 Presence of polyubiquitinated protein and Aggresomes formation

Protein aggregates are usually present as amyloid accumulation in cardiomyocytes of patients with DCM. We wanted to check whether there is any evidence that would support the hypothesis of a failure of the UPS system.
As shown in **Figure 4.3**, the hearts of patients with DCM are characterized, compared to controls, by accumulation mainly in cardiomyocytes, of polyubiquitinilated protein.
These protein aggregates are segregated from the cell in perinuclear regions to form the aggresomes, so we investigated whether any their components are detectable in cardiomyocytes of patients with DCM.

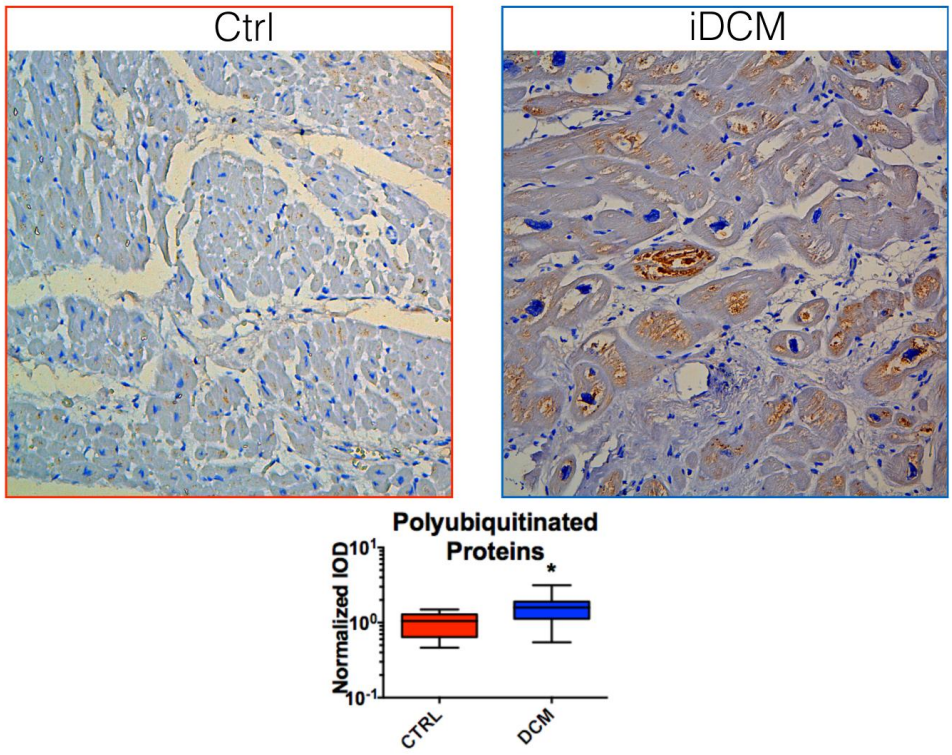


Figure 4.3: Immunohistochemistry staining on myocardial samples obtained by healthy controls and patients affected by iDCM which show polyubiquitinated proteins accumulation within cardiomyocytes. Histograms below show quantitative evaluation of integrated optics between samples and controls.Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

We have observed the presence of intracellular aggresomes in cardiomyocytes of patient affected by iDCM and the colocalization of p62 and vimentin in these aggresomes, as shown in **Figure 4.4**

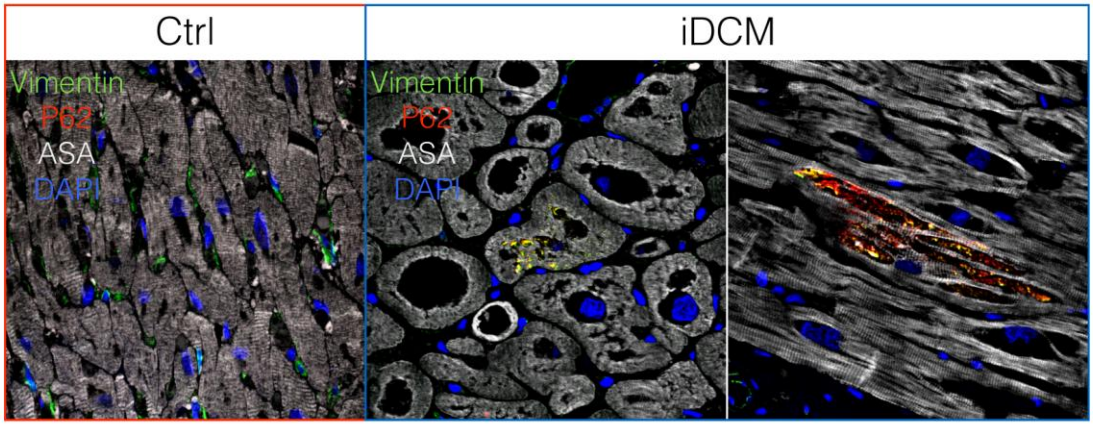


Figure 4.4: Confocal images which show aggresomes within cardiomyocytes of patients affected by iDCM. In control heart, vimentin stain in particular interstitial cells, in patients affected by iDCM we observed intracellular polyubiquitinated proteins aggregates enclosed by a network of vimentin.

4.4 Altered Autophagic Flow in Patients Affected by iDCM

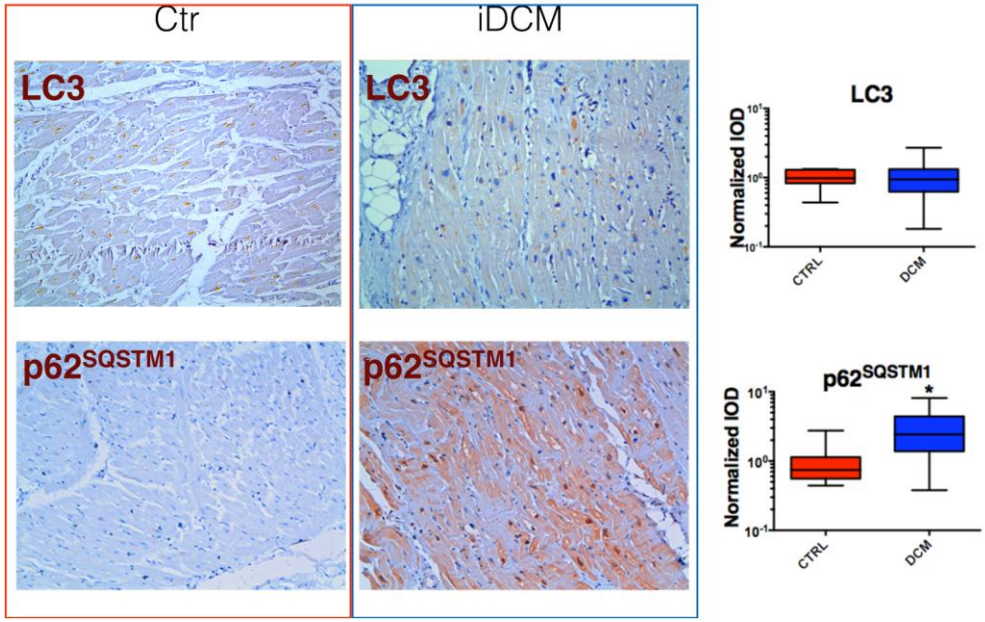


Figure 4.5: Immunohistochemistry staining for LC3 and p62/SQSTM1 on control hearts and hearts of patients affected by iDCM. The panels on the right show results of quantitative evaluation of integrated optics between samples and controls.Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates $p<0,05$ between two groups.

As shown in literature, aggresome stimulates autophagy so we wanted evaluate whether, in the cases analyzed, it is possible to mark elements indicative of abnormalities of the Autophagy Lysosomal Pathway (ALP).
We analyzed the presence of punctae LC3 and the levels of p62 in the controls and in patients affected by iDCM. In the presence of a normal autophagic flow, an increase in the levels of LC3 corresponds to a reduction in the levels of p62, given by the degradation of this receptor for elements ubiquitinated by ALP.
In our study, we observed similar levels of LC3 and we found an accumulation of cytoplasmic p62 (**Figure 4.5**). This seems to indicate a positive feedback on the system ALP insufficient to dispose of the accumulation of ubiquitinated proteins.

4.5 Mitochondria dysfunction accumulation

Arrest of the autophagic / lysosomal pathway can lead to accumulation of dysfunctional mitochondria within the cell.
To check if in patients affected by iDCM was present this phenomenon, it was evaluated mitochondria expressing Parkin1, a protein involved in the pathogenesis of Parkinson's which is stabilized on the outer membrane of mitochondria whose membrane potential (ΔΨ) is dissipated.

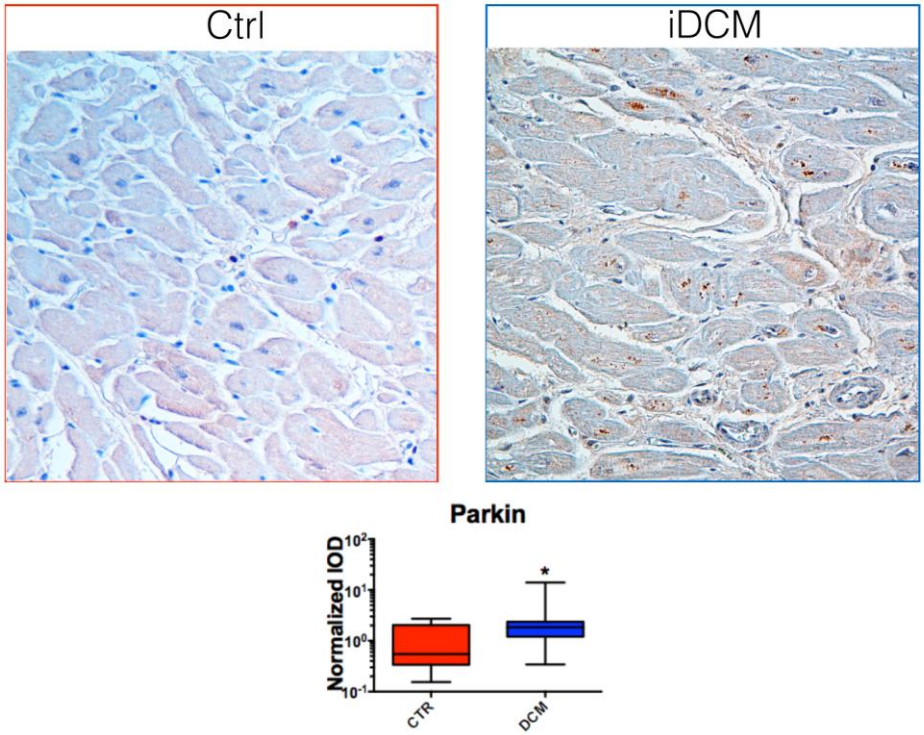


Figure 4.6: Immunohistochemistry staining for Parkin1 on control hearts and hearts of patients affected by iDCM. Histograms below show quantitative evaluation of integrated optics. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

As shown in **Figure 4.6**, we have evaluated an increase of Parkin1 levels in patients affected by iDCM. Colocalization analysis showed that the reactivity was mainly confined to the mitochondria.

4.6 4-Hydroxynonenal (HNE)

4-hydroxynonenal (HNE) s one of the major products of lipid peroxidation derived from the oxidation of polyunsaturated fatty acids ω-6, such as linoleic acid, γ-linolenic acid or arachidonic acid.
Cardiolipin, class of membrane phospholipids containing 3 skeletons of glycerol and 4 side chains of fatty acid predominantly localized in the mitochondrial inner membrane, seems to be one of the main sources of 4-HNE so it is believed that levels of 4-HNE intracellular reflect a state of mitochondrial dysfunction.
We stained, by immunohistochemistry with primary antibody anti-4HNE, heart samples of patients affected by iDCM to evaluate whether there is a change in the formation and accumulation of intracellular HNE.

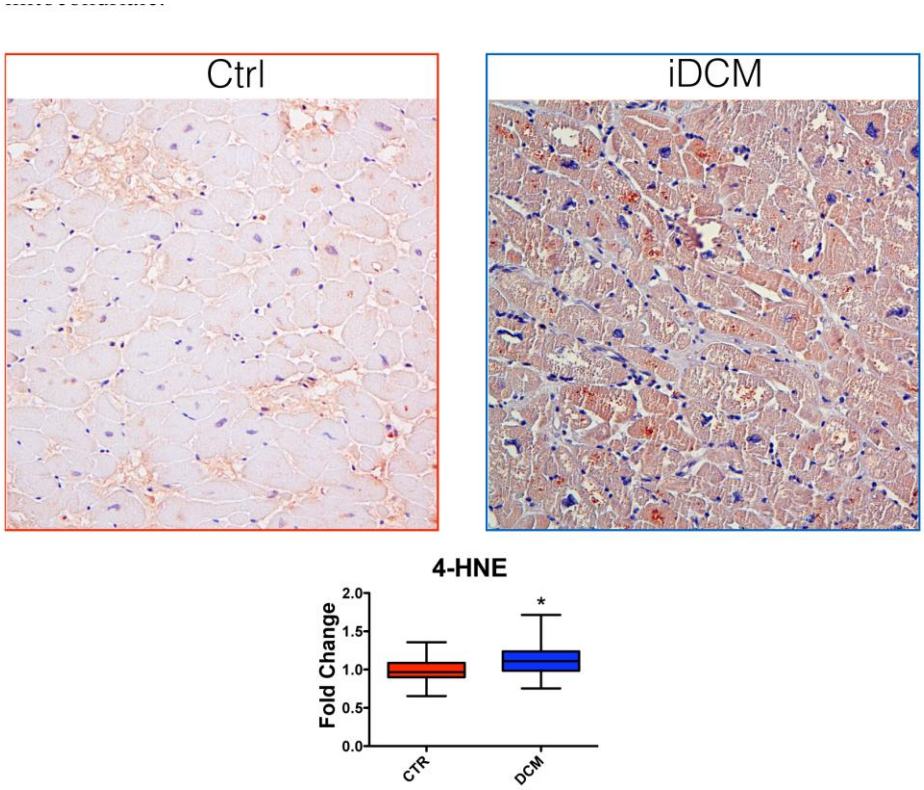


Figure 4.7: Immunohistochemistry staining for 4-HNE on control hearts and hearts of patients affected by iDCM. Histograms below show quantitative evaluation of integrated optics. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

As shown the two images in **Figure 4.7**, the control heart show a reactivity less significant than the heart obtained from patient with iDCM. In particular, in the patients, the positivity appears mainly localized to the cytoplasm of cardiomyocytes and has a granular appearance, often perinuclear. This suggests a perinuclear localization to the mitochondria.
The results obtained demonstrate that the hearts of patients with iDCM are characterized by accumulation of products of lipid peroxidation. This suggests that they are characterized by an alteration in the autophagic flow with consequent accumulation of dysfunctional mitochondria.

4.7 NLRP3 and CASPASE-1

The accumulation of dysfunctional mitochondria is associated with activation of inflammasome so we studied, by immunofluorescence, its functionality.
Infammasome integrates signals of cellular damage (DAMPs) and infection (PAMPs/ MAMPs) to translate them into signals and answers involving the processing and release of cytokines inflammatory (as IL1β and IL18), cell immune activation and cell death (pyroptosis). Following activation, the protein ASC moves from the nucleus to the cytoplasm where it is assembled with the receptors, as NLRP3 (nucleotide binding oligomerization domain-NOD-like receptor P3), in a structure that recruits and actives pro-Caspasi1 and pro-interleuchina1β.
To assess the state of inflammasome activation, we stained samples for NLRP3, caspase1 and α-sarcomeric actin. Subsequently, we quantified the colocalization of the first two markers, both in myocytes that in interstitial cells.

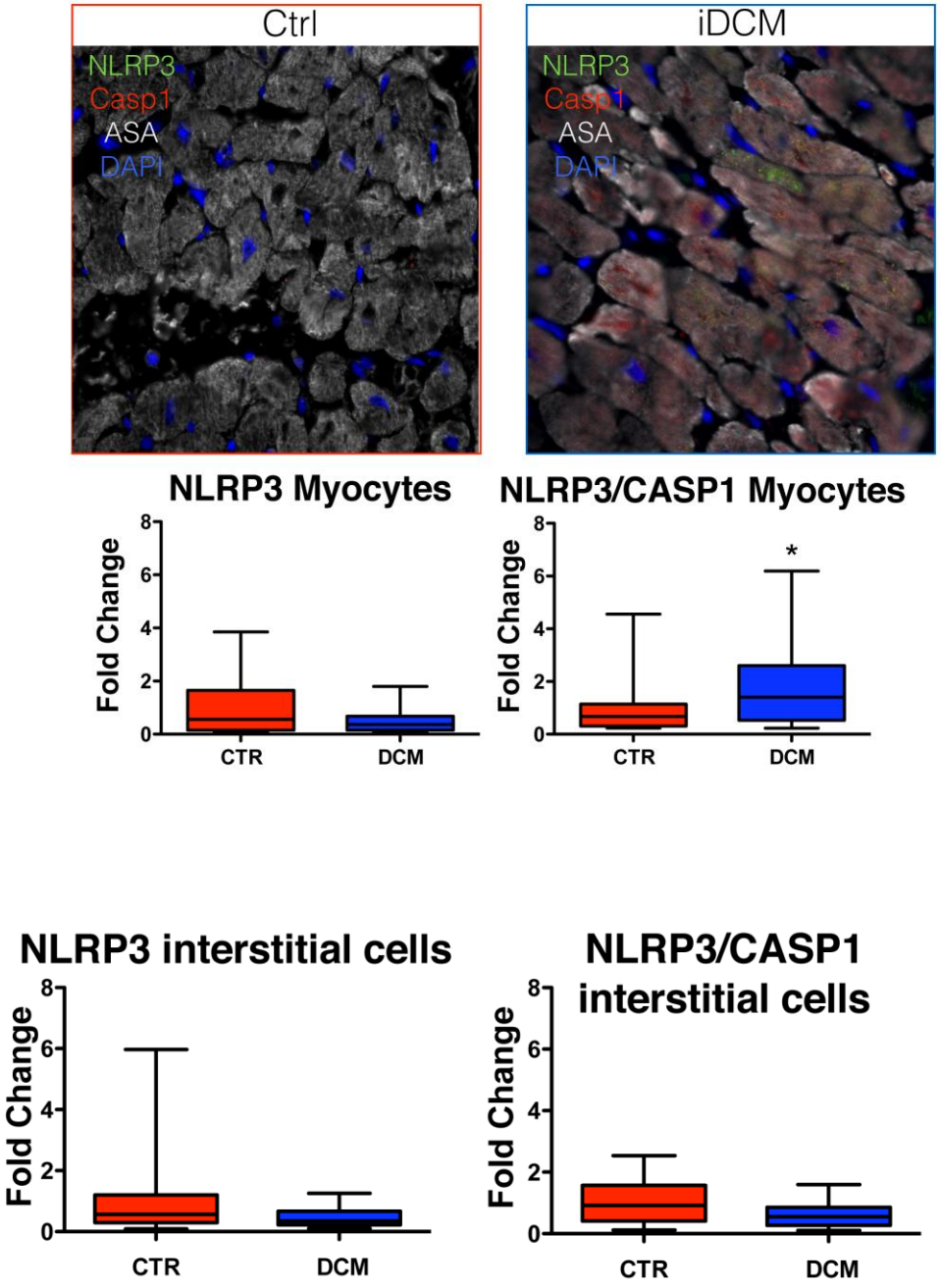


Figure 4.8: Fluorescence staining for NLRP3 (green), Caspase1 (red) and α-sarcomeric actin (white) on control hearts and hearts of patients affected by iDCM. Histograms below show quantitative evaluation of integrated optics. Data are

represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

As shown in **Figure 4.8**, there were not significant differences in the amount of NLRP3 in myocytes between patients and controls but myocytes of patients showed levels of colocalization NLRP3/Capase1 significantly higher than controls. On the contrary, the interstitial cells of patients and controls not differ in the levels of NLRP3, and in NLRP3/Caspase1 colocalization.
Taken together, the data suggest that the hearts obtained from patients affected by iDCM are characterized by inflammasome activation. It is important to note that this phenomenon affects primarily cardiomyocytes, underling the importance of proteostasis in these cells.

4.8 P53 binding protein1 (53BP1)

Cells characterized by mitochondrial dysfunction subjected to oxidative stress activate a DNA Damage Response (DDR). The DNA damage response (DDR) is a complex signaling network including cell cycle checkpoints, DNA repair and damage tolerance pathways. The DDR is affected by myriad cellular components and processes, which include chromatin structure, DNA replication, telomeres, cell growth and cell-cycle status. Failure of the DDR or associated events causes genomic instability, an underlining cause of several human syndromes and it also associated with various age-related diseases, particularly cancer.

To check whether, in patients affected by iDCM, is possible demonstrate this phenomenon, we evaluated a key molecule in response to DDR. This molecule, called P53 binding protein1 (53BP1), is an important regulator of the DDR that promotes, with

BRCA1, the chromatin remodeling needed to repair the damage. It is recruited in areas where histone H2AX is phosphorylated by kinases such as ATM.

To quantify the frequency which myocytes and interstitial cells are found in a state of DDR, in patients affected by iDCM and in the controls, we stained our samples with anti-53BP1 and anti-α sarcomeric actin antibodies and we evalueted 53BP1 nuclear positivity (**Figure 4.9**).

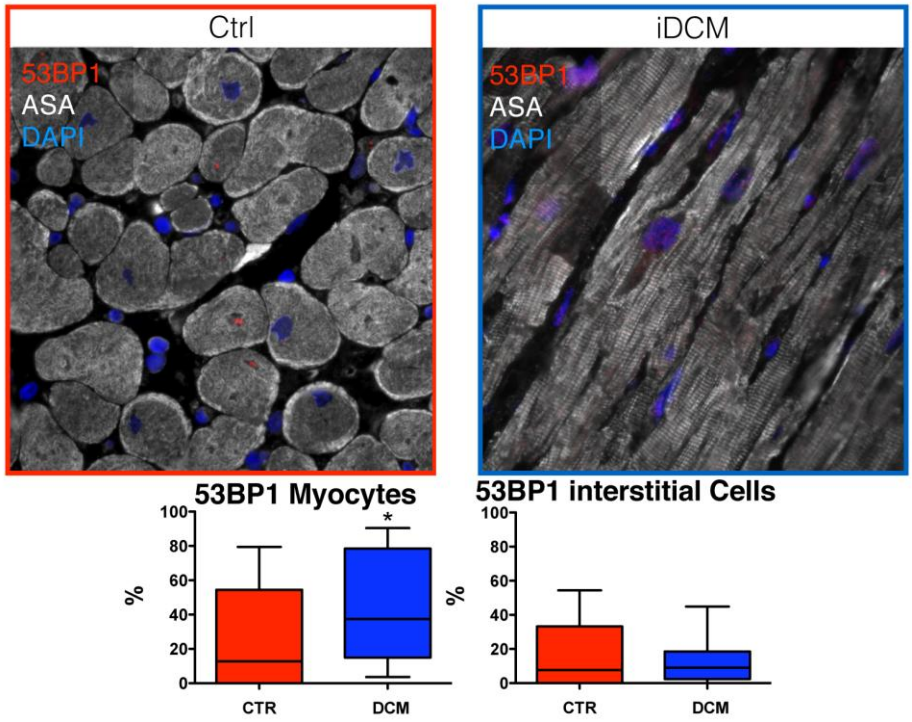


Figure 4.9: Fluorescence staining for 53BP1 (red) and α-sarcomeric actin (white) on control hearts and hearts of patients affected by iDCM. Histograms below show quantification of frequency of myocytes and interstitial cells which show nuclear positivity for 53BP1. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

The results obtained demonstrate that, although the amount of interstitial cells expressing 53BP1 not differ between patients and controls, a significantly greater fraction of myocytes 53BP1 positive characterize the hearts of patients with DCM. Our results suggest that cellular senescence vicious specifically affects the myocytes of patients with iDCM.

4.9 Nuclear Factor-kappaB (NF-kB)

A typical characteristic of senescent cells is the activation of a program that leads to the production of a secretome rich in pro-inflammatory cytokines, pro-angiogenic factors and matrix remodeling enzymes. This program is called Senescence Associated Secretory Phenotype (SASP). One of the most important transcription factors (with C/EBPβ) for transcriptional activation response SASP is NF-kB [69].

The activation of NF-kB is carried out for the phosphorylation of the inhibitory protein IkB, followed by nuclear translocation of p65 RelA, mediated by IKKαβ complex.

To check whether this view is activated in patients affected y iDCM, we stained our samples with an antibody th.at recognizes p65 RelA subunit.

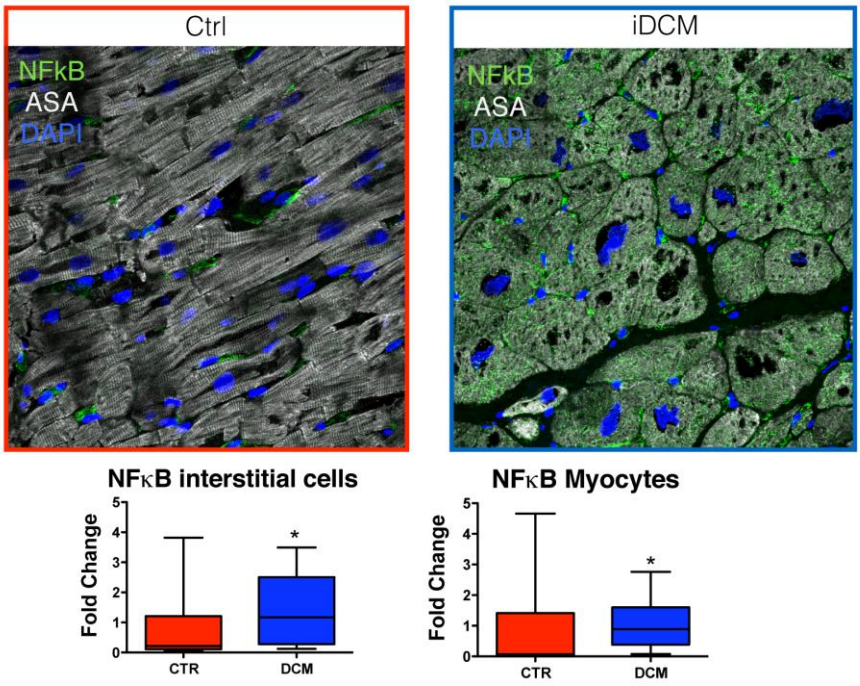


Figure 4.10: Fluorescence staining for NF-kB (green) and α-sarcomeric actin (white) on control hearts and hearts of patients affected by iDCM. Histograms below show quantification of frequency of myocytes and interstitial cells which show positivity for NF-kB. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

As shown in **Figure 4.10**, our samples are characterized by high antibody reactivity. Hearts of patients affected by iDCM are characterized, compared to controls, greater positivity both cytoplasmic and nuclear. In this case, differences observed concern myocytes and interstitial cells. Our results suggest that in the hearts of patients with iDCM present there is activation of an answer compatible with the SASP.

4.10 Cardiomyocyte Hypertrophy

NF-*k*B is involved also in cardiomyocyte hypertrophy and we checked whether also myocytes of patients affected by iDCM are characterized by indexes of hypertrophy such as an increase of the area of the transverse section and of greater diameter (length). Morphometric analysis of these two parameters, performed on histological sections stained with anti- α-sarcomeric actin antibody, showed that, compared with healthy controls, the myocytes of patients with iDCM appear enlarged in both dimensions **(Figure 4.11)**.

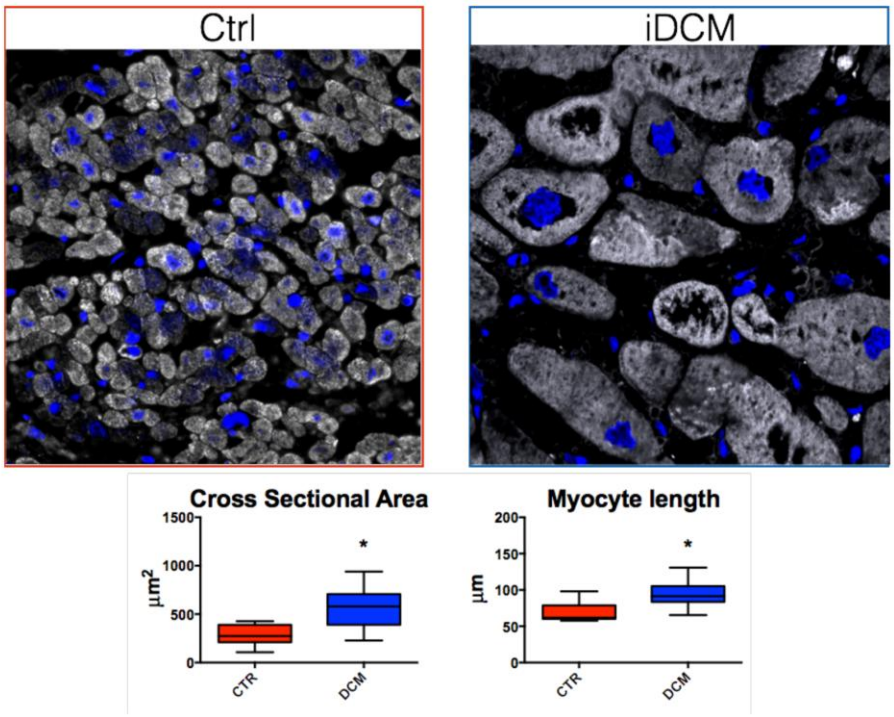


Figure 4.11: Histograms summarize the results of the quantification of the cross-sectional area and length myocyte. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

These results suggest that pro-inflammatory conditions triggered by the vicious circle of senescence actives cell growth processes, stimulating protein synthesis and further exacerbating the deterioration of proteostasis.

4.11 Correlation between Parameters Analyzed

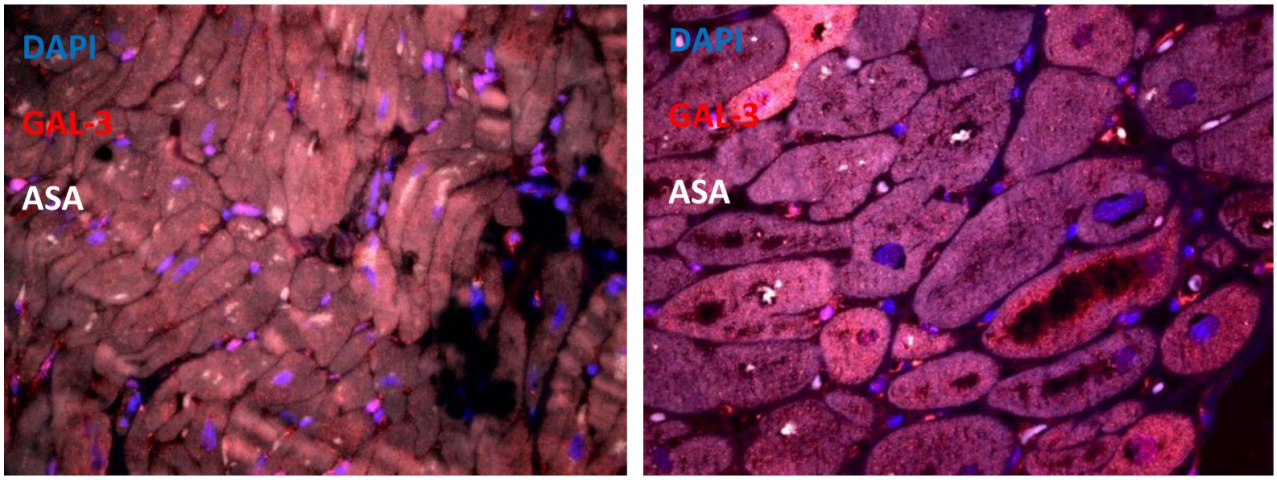
To support a possible association between the events previously described, we assessed whether, within the population of patients affected by IDCM, the parameters that we analyzed are correlated. The correlation analysis, carried out with Pearson test, has shown that there is a significant association between levels of NLRP3/Caspase1 in myocytes and those of 53BP1 in myocytes and not (p=0,419, p=0,007 e p=0,298; p=0,044, respectivily) and with NF-kB levels (p=0,316, p=0,029) not in myocytes.

Our results suggest that a vicious circle is to be created in myocytes and in interstitial cells.

4.12 Lysosomal Membrane Permeabilization

Galectin-3

A characteristic of cells that undergo to senescence is the lysosomal membrane permeabilization, we studied if these evidences are present also in human hearts explanted by patients affected by iDCM. We evalueted the presence of punctae of galectin3 in myocytes of patiens and donors.



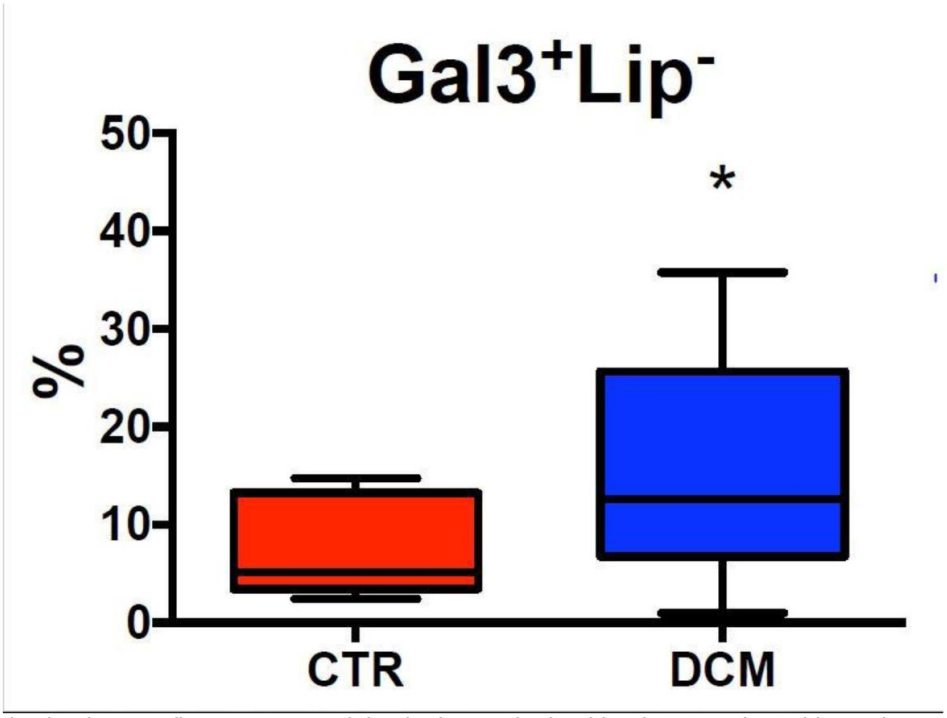


Figure 4.12 Galectin3 immunofluorescence staining in tissue obtained by heart explanted by patients affected by iDCM and by donors. Histogram represents the results obtained by protein quantification. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

These data demonstrate that heart of patients affected by iDCM present an increase of punctae of Galectin3 in the cytoplasm (there is not colocalization with lipofuscins -Lip-). This result indicates an increase of lysosomal permeability in heart of patients affected by iDCM.

4.13 WESTERN BLOTTING

To verify the data obtained by immunohistichemistry about autophagic flux alteration in patients affected by iDCM, we have analyzed proteins involved in the regulation of autophagy by Western Blotting.

Protein extracts were obtained by 6 atria by patients affected by iDCM and 6 atria by donors and we analyzed:

- Beclin-1 (Molecular Weight 60 kDa);
- Atg3 (Molecular Weight 40 kDa);
- Atg7 (Molecular Weight 78 kDa);
- Lc3-II (Molecular Weight 14 and 16 kDa);
- Actin (Molecular Weight 43 kDa).

Polyclonal anti-actin antibody was used to normalize the protein content in the total extracts.

4.13.1 Beclin-1

Beclin-1 has a central role in autophagy. It is expressed in many human and murine tissues, and is localized primarily within cytoplasmic structures, including the ER, mitochondria and the perinuclear membrane.
In Figure 4.13 is shown a representative WB of proteins extracted by atria of donors (C1, C2, C3) and by patients affected by iDCM (DCM1, DCM2, DCM3). The optical density data of the champions were normalized with actin.

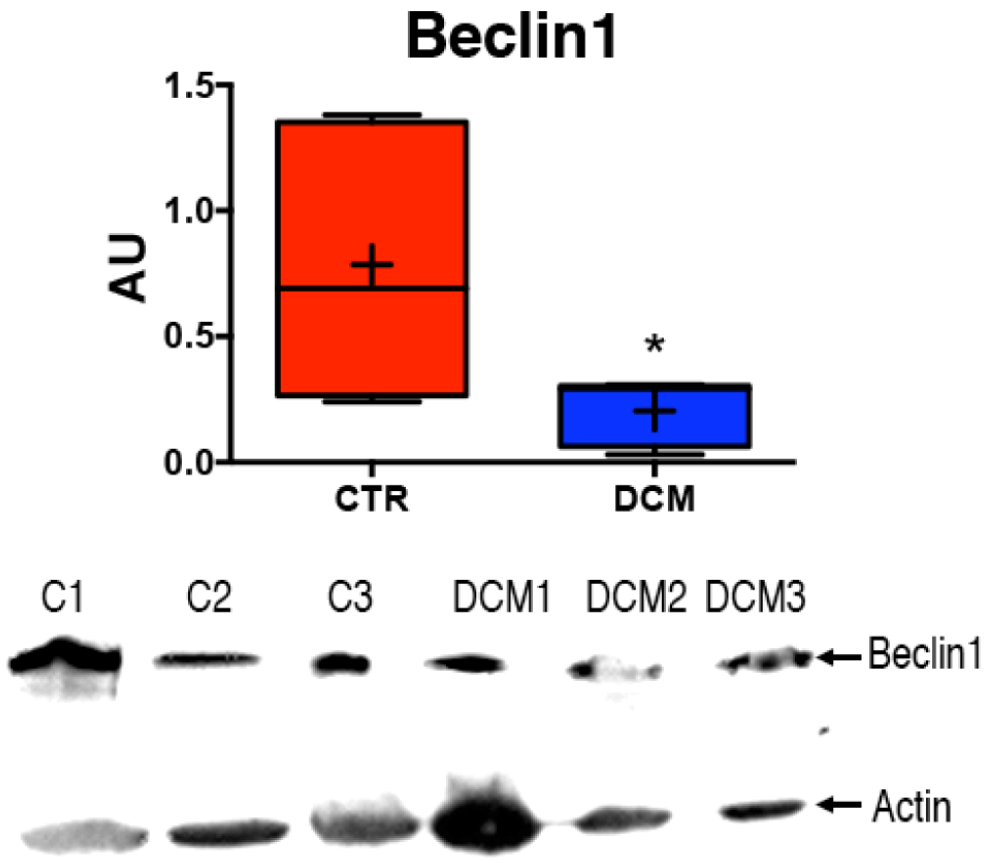


Figure 4.13 Western Blot of Beclin-1. The Histogram represents the results obtained by protein quantification. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

These results suggest that protein extracts from patients affected by iDCM show a beclin-1 increase respect to donors, pointing out a condition of instability and autophagy malfunction in cells derived from pathological hearts.

4.13.2 ATG3

ATG3 is a involved in protein ubiquitination anticipating autophagic process.
ATG3 is fundamental for conjugation of ATG12 and ATG8 proteins important for autophagic vesicles formation. ATG3 is responsible for the E2-like covalent binding of phosphatidylethanolamine to the C-terminal Gly of ATG8-like proteins (GABARAP, GABARAPL1, GABARAPL2 or MAP1LC3A); this complex is important for the autophagic process.
ATG3 present two different regions, one of which is responsible for binding with ATG7, an enzime “E1-like” in turn responsible for binding with ATG8.
In Figure 4.14 is shown a representative WB of ATG3 by atria of donors (C1, C2, C3) and by patients affected by iDCM (DCM1, DCM2, DCM3). The optical density data of the champions were normalized with actin.

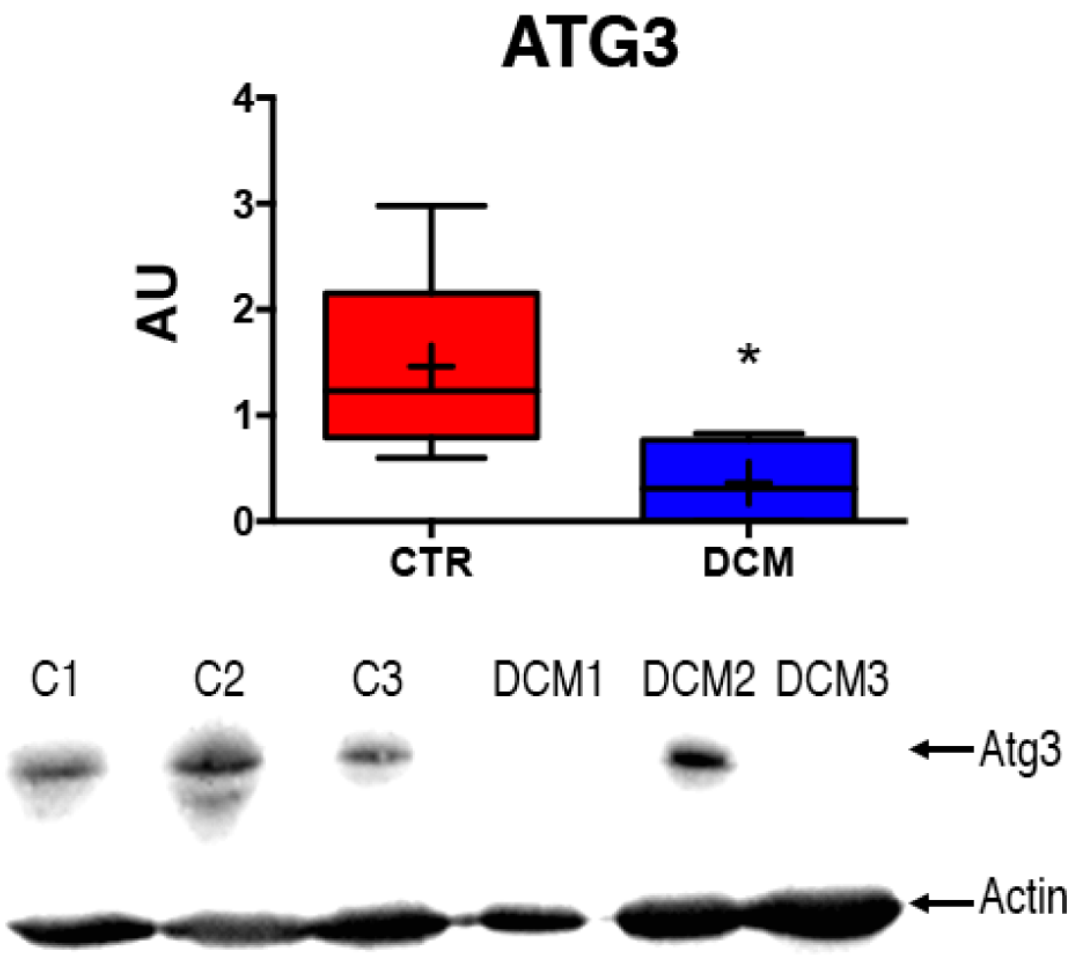


FIGURE 4.14 Western Blot of ATG3. The Histogram represents the results obtained by protein quantification. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

These data show a reduction of ATG3 levels in protein extracts of cells derived by patients affected by iDCM respect to the donors suggesting further evidence of autophagy insufficiency in the patients.

4.13.3 ATG7

ATG7 is a protein involved in autophagic process, as ATG3.
ATG7 mediates the conjugation reaction between ATG12 and ATG5, proteins involved in autopagy molecular mechanisms.
In particular, ATG7 and ATG5 are fundamental for macroautophagy in mammals. It has been proven as mouse cells without ATG5 and ATG7 can still form autophagosomes and autolysosomes and perform the degradation of proteins by autophagy, when subjected to certain stress factors.
In Figure 4.15 is shown a representative WB of ATG7 by atria of donors (C1, C2, C3) and by patients affected by iDCM (DCM1, DCM2, DCM3). The optical density data of the champions were normalized with actin.

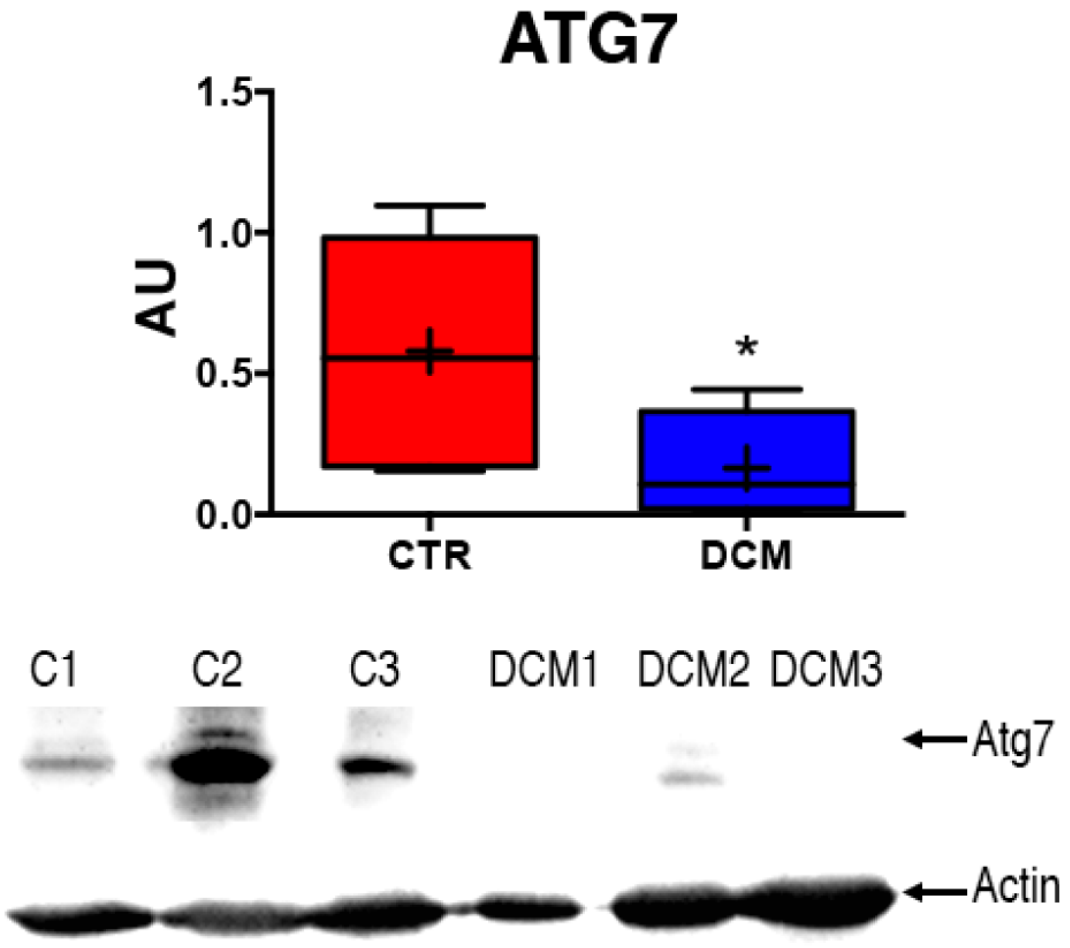


FIGURE 4.15 Western Blot of ATG7. The Histogram represents the results obtained by protein quantification. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

These data show a reduction of ATG7 levels in protein extracts of cells derived by patients affected by iDCM respect to the donors suggesting further evidence of autophagy insufficiency in the patients.

4.13.4 LC3-II

(LC3) is a soluble protein with a molecular mass of approximately 17 kDa that is distributed ubiquitously in mammalian tissues.

During autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are degraded by lysosomal hydrolases. At the same time, LC3-II in autolysosomal lumen is degraded. Thus, LC3-II is used as indicator of autophagic activity [102].

In Figure 4.16 is shown a representative WB of ATG7 by atria of donors (C1, C2, C3) and by patients affected by iDCM (DCM1, DCM2, DCM3). The optical density data of the champions were normalized with actin.

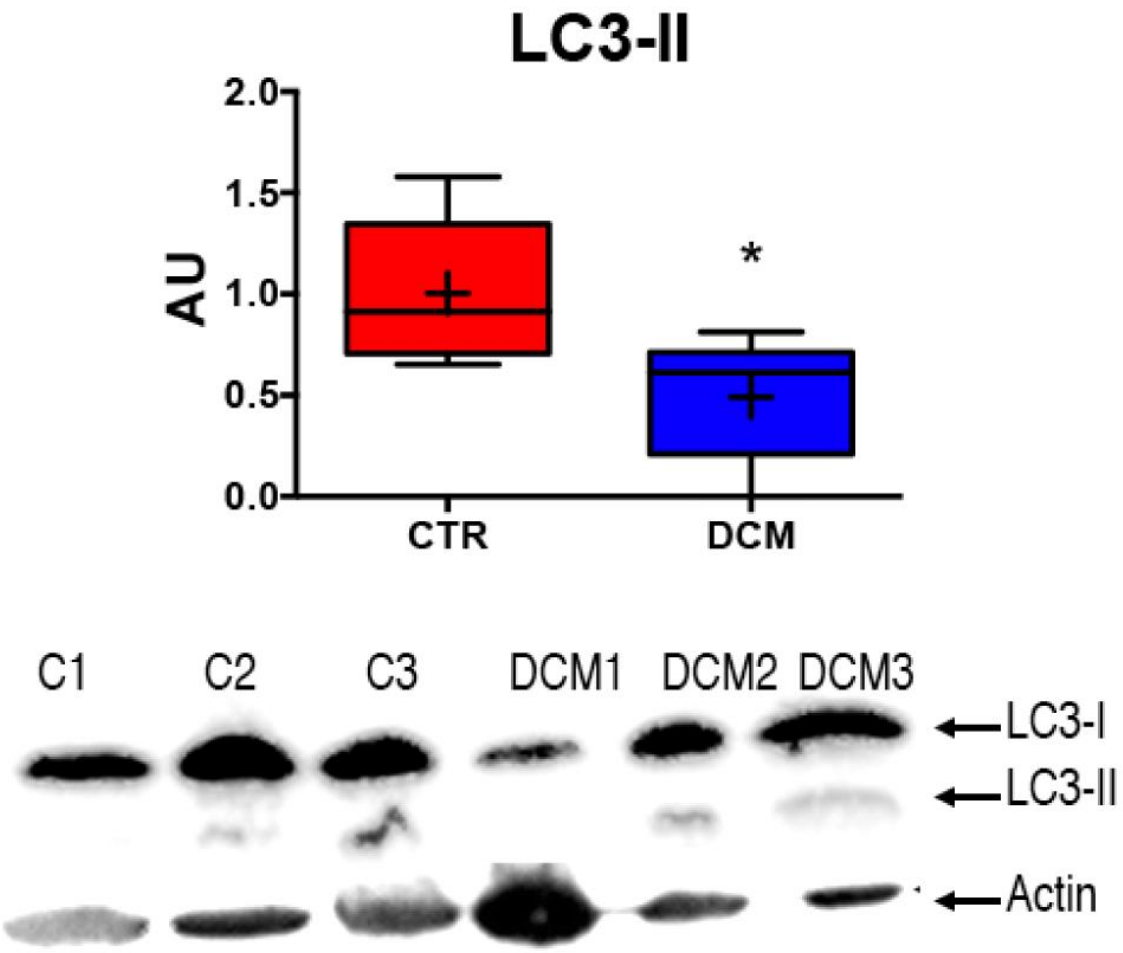


FIGURE 4.16 Western Blot of LC3-II. The Histogram represents the results obtained by protein quantification. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

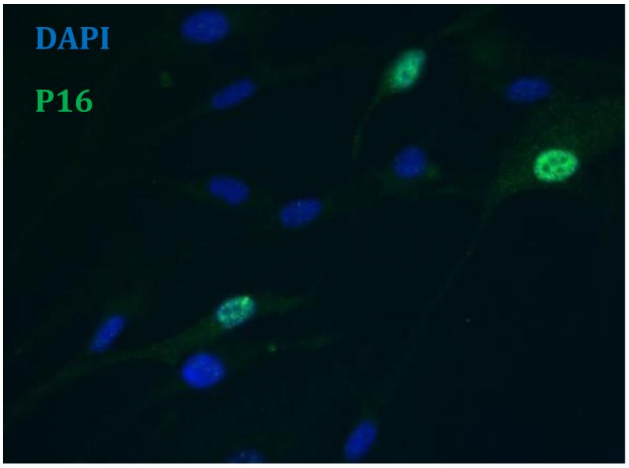
These data show a reduction of LC3-II levels in protein extracts of cells derived by patients affected by iDCM respect to the donors suggesting further evidence of autophagy insufficiency in the patients.

4.14 IMMUNOFLUORESCENCE

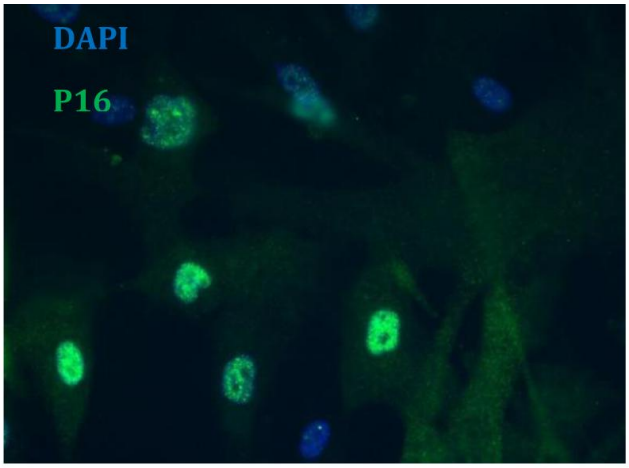
We studied cardiac stem cells evaluating if heart disease is accompanied in senescence as observed in ischemic cardiopathy

4.14.1 $p16^{INK4A}$

We evaluated the expression of p16INK4A in D-CSC (Cardiac Stem Cells by Donor) and E-CSC (Cardiac Stem Cells by Explanted hearts) by immunofluorescence. $p16^{INK4A}$, localized mainly in nucleus, is a cyclin-dependent kinase inhibitor and so blocks cellular cycle and mitosis.
For this study, $p16^{INK4A}$ was used as senescence marker.



D-CSC



E-CSC

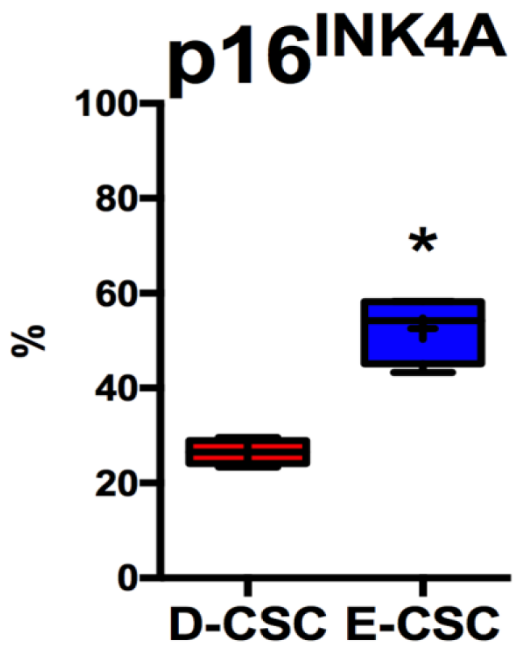


FIGURE 4.17 $p16^{INK4A}$ on cells obtained by donors hearts and explanted hearts by patients affected by iDCM. Histogram represents the results obtained by protein quantification. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

These data suggest that cells obtained by patients affected by iDCM show an increase of $p16^{INK4A}$ suggesting an inibition of cellular cycle and the mitotic process.

4.14.2 Ki67/γH2AX

Cellular senescence is a stable arrest of the cell cycle in response to DNA damage.
We made a double staining ki67/γH2AX to verify if DNA damage is inversely correlated to cell proliferation.
Ki67 is a nuclear protein associated to cell proliferation so it is considered as cell growth marker. γH2AX is a product of H2AX Histone phosphorylation, after a damage to double stranded DNA, within nucleosomes.
As shown in Figure XXX, we observed that the expression of ki67 is increased in D-CSC (Cardiac Stem Cells by Donor) respect to E-CSC which show an increase of γH2AX

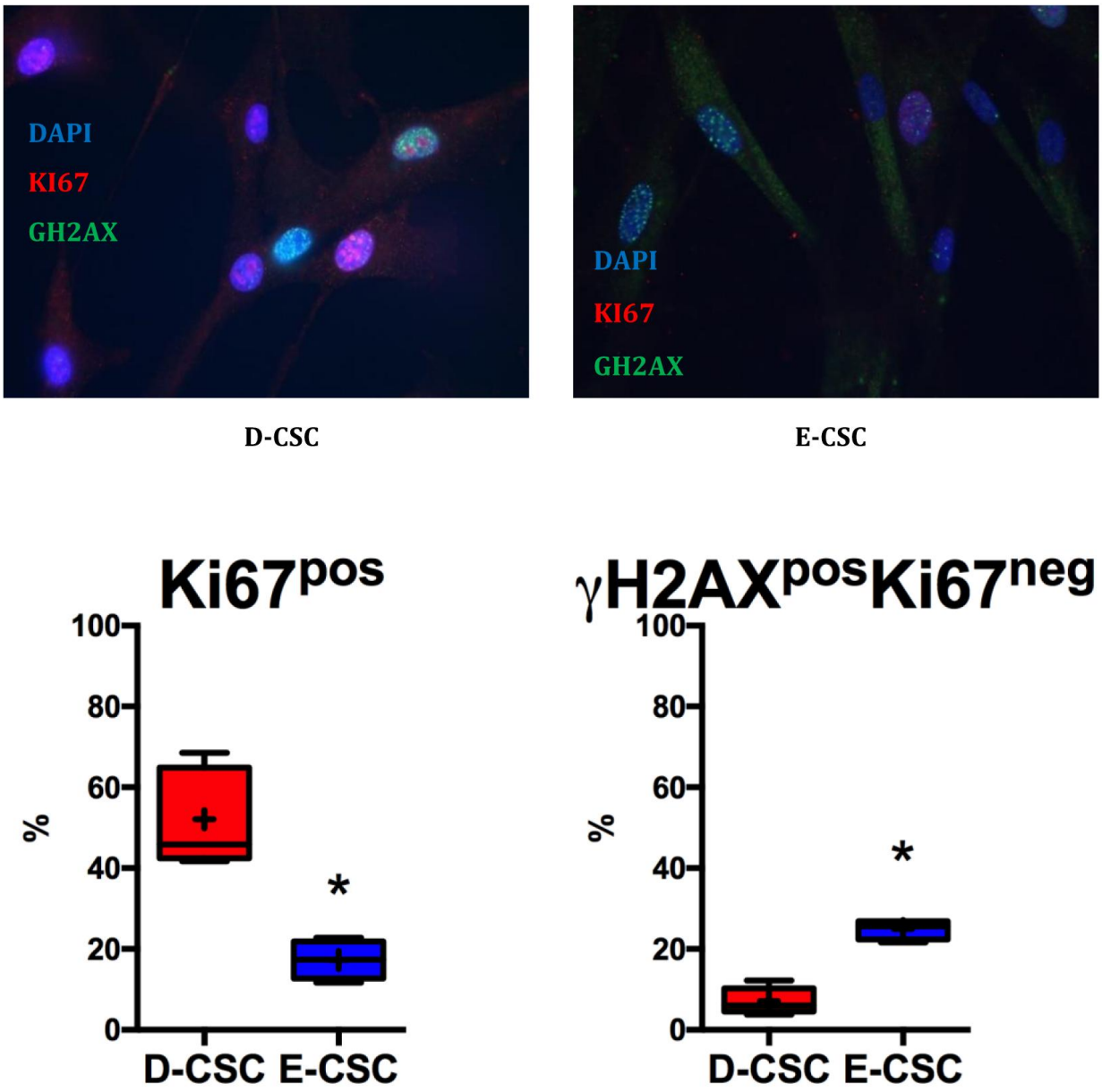


FIGURE 4.18 ki67/γH2AX immunofluorescence staining on cells obtained by donors hearts and explanted hearts by patients affected by iDCM. Histogram represents the results obtained by protein quantification. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

These data suggest that cells from patients affected by iDCM show DNA damage, non associated to cell proliferation, as consequence of senesce state that identifies pathological state.

4.14.3 Galectin-3/LAMP2

Lysosomes promote the elimination of autophagosoma products. A central role is played by lysosomal membrane that keeps the organelles in conditions useful to carry out their function; It was demonstrated that events of lysosome membrane permeabilization (LMP) are associated to malfunction of the autophagic-lysosomial pathway and next tissue degeneration, condition which can determines pathologies as DCM.
Galectin-3 is best suited for LMP detection. Punctae of galectins absent in healthy cells and their formation takes place regardless of how the lysosome has been damaged: this

suggests that the translocation of them to the lysosome is a general consequence of the rupture of the lysosomal membrane.
To verify LMP detection, we stained cells with Galectin-3/LAMP2 to identify lysosomes damage in presence of punctae of galectin-3 and to localize lysosomes through protein associated to LAMP membrane.

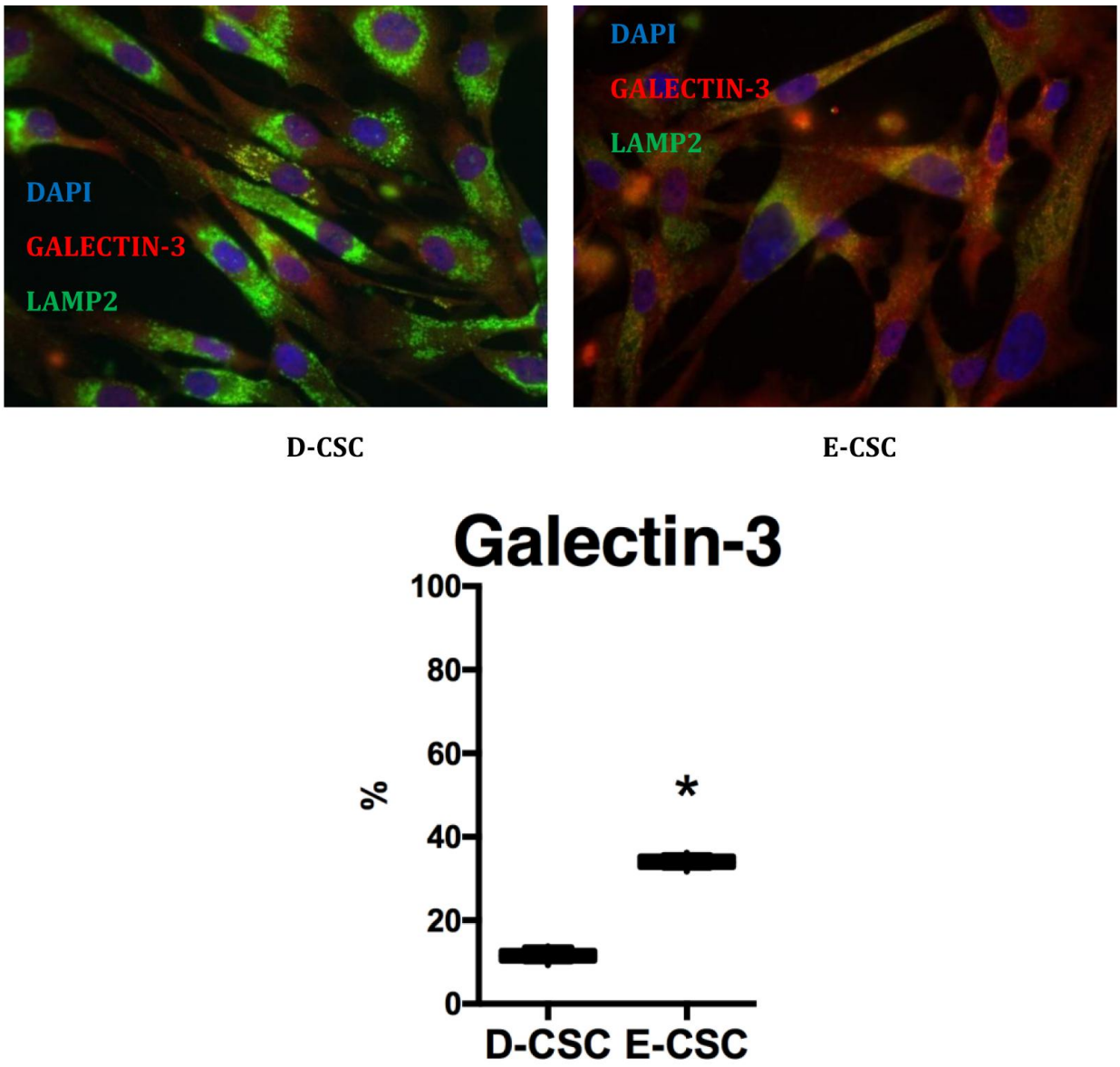


FIGURE 4.19 Galectin3/LAMP" immunofluorescence staining on cells obtained by donors hearts and explanted hearts by patients affected by iDCM. Histogram represents the results obtained by protein quantification. Data are represented as median (line), interquartile range (box), 5° and 95° percentile (bars). Asterisk indicates p<0,05 between two groups.

These results demonstrate that cells obtained from patients heart present an increase of punctae of galectin-3 (red) in lysosomes (green), indicating that CSCs by patients affected by iDCM present lysosomal damage and membrane permeabilization.

4.14.4 PARKIN1

An arrest of the autophagic/lysosomal pathway can lead to accumulation of dysfunctional mithocondria within the cell. In particular, the increase of ROS production can determine

the accumulation of oxidate proteins as aggregates that interfere with cell function. ROS determine also mutations in mitochondrial DNA and damage to respiratory enzymes. It is important that there is a correct regulation of mitophagia to eliminate damaged organelles. A protein involved in the marking of mitochondria for autophagia is Parkin, a E3 ubiquitin ligase localized in the mitochondrial external membrane.
To test if there is an accumulation of mitochondria for autophagia in CSC obtained by heart of patients affected by iDCM, we studied the co-localization of Parkin1 in mitochondria.
We noted morphological differences between donors and patients mitochondria. Mitochondria of donors are smaller and point, while mitochondria of patients are longer and branched

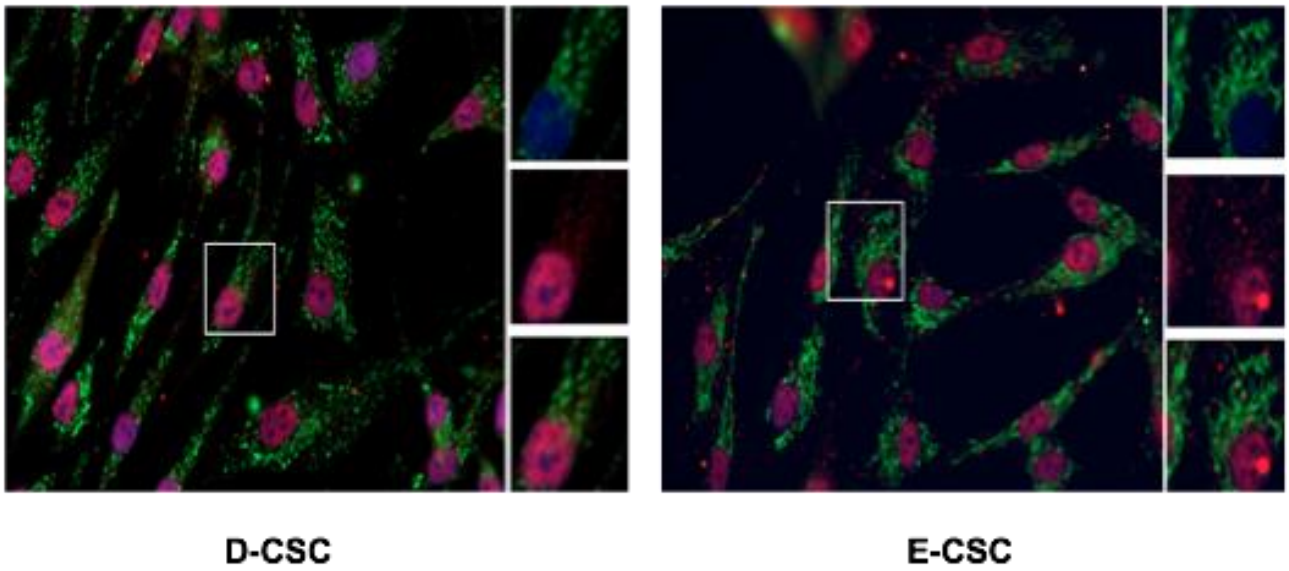


Figure 4.20 Parkin1 immunofluorescence on mitochondria obtained by cells of donors heart and patients heart. Nuclei are stained in blu, Mitochondria in green and Parkin in red.

5 - MATERIALS AND METHODS

5.1 PATIENTS ENROLLED AND TISSUE SAMPLING

In this study we analyzed 48 explanted hearts of patients with idiopathic dilated cardiomyopathy, affected by terminal heart failure and transplanted between June 1993 and January 2012 at the Udine University Hospital section of Cardiac Surgery. As a control, we used 11 hearts collected during the autopsy of patients who died for no cardiovascular causes and 7 hearts of potential organ donors is not used for transplantation.
The hearts obtained, once described macroscopically by the pathologist, are immersed in formalin in a container for surgical specimens sealed with a hermetic cap ready to undergo a fixing process that varies from 24 to 72 hours (after the first 24 hours, based on a macroscopic evaluation, the pathologist decides whether an organ should remain other 24-48 hours in the same formalin). Once the heart is fixed, the pathologist pick up samples measuring about 1.5 x 1 x 0.5 cm obtained from: left ventricle, front ventricle, side ventricle, posterior ventricle, septum, right ventricle, right and left atria. These samples, placed in suitable containers for bioptic material (called histological cassettes), are placed in a new container for surgical specimens full of formalin, and so ready to processing, inclusion and section.
The study, in accordance with the Declaration of Helsinki, was approved by Ethics Committee of Udine (2 August 2011, reference number 47831) and written informed consent was obtained from each patient.

5.2 CARDIAC STEM CELLS ISOLATION AND IN VITRO EXPANSION

Atrial samples were collected both from donor hearts and from explanted hearts of patients affected by idiopathic dilated cardiomyopathy undregoing cardiac transplantation at the University Hospital of Udine.
Human Cardiac Stem Cell lines (CSCs) were isolated, as in [96, 97]. Specifically, isolation was performed by washing atrial fragments in Basic Dissociation Buffer (BDB) to eliminate blood from tissue. Adipose tissue and macroscopically apparent areas of fibrosis were removed. The tissue underwent mechanic disaggregation with scalpels and scissors until fragments have a dimension not larger than 1 mm3. The tissue was then washed with BDB and the cell solution was centrifuged 5 minutes at 500g and supernatant discarded. The pellet was enzimatically dissociated by incubation in a 0.25% Collagenase type II solution (Worthington Biochemical Corporation). The enzyme/cell solution was incubated for 15-20 minutes at 37°C in a tube rotator. Collagenase activity was stopped by the addition of 0.1% bovine serum albumin (BSA, Sigma-Aldrich) solution in BDB. Cell suspension was first centrifuged at 100g for 1 minute to remove myocytes and subsequently at 500g for 5 minutes. The resulting pellet was resuspended in 1 ml of BDB and filtered through a pre-wet stainer (BD Falcon) in order to select a population less than 40 µm in diameter. The filtered suspension was centrifuged at 500g for 5 minutes. The supernatant was discarded and cell pellet was resuspended in 1 ml of Human MeseCult Proliferation Medium (STEMCELL Technologies). Cells were cultured in the presence of 100U Penicillin and 100µg/L Streptomycin (Life Technologies). Cells were plated at a concentration of 1.5 milion in a 100-mm dish and mainteined at 37°C, 5% CO2.

5.2.1 DETACHMENT AND EXPANSION OF CSCs

To detach cells from substrate, the plate was washed twice with 5ml of HBSS. 2ml of TrypLE Express solution (Gibco- Life Technologies) was added to the cells and incubated

at 37°C, until the cells were observed to dissociate, after which 7ml HBSS were added to the cells to inhibit enzymatic activity. Cell suspension was centrifuged at 500g for 5 minutes and the supernatant was discarde. The cell pellet was resuspended in 1ml of culture medium. The cells were plated at a density of 5,000 cells/cm2. After the second passage, the tissue culture was coated with 10 µg/ml human Fibronectin (Sigma-Aldrich) in HBSS and medium was switched to expansion medium.

5.3 SAMPLES HEART FIXING AND PROCESSING (Dehydration, Clearing and Infiltration)

To prevent biological tissues undergo decomposition, oxidation and degradation of its chemical-physical characteristics, samples, picked up from the patients, must be treated with fixatives. Inadequate fixation leads to degradation of the sample.
In the study proposed in this thesis, it was used neutral formalin (3%-5%, formaldehyde 1%-3% methanol, buffered in aqueous solution at pH 7,2-7,4). Formalin is a powerful antibacterial substance, which avoids the putrefaction of biological tissues and gives the sample adequate mechanical characteristic for its processing.
The formalin has a high degree of penetration and does not cause excessive tissue hardening and does not dissolve the lipids, because it is a non-coagulating fixative chemical additive, where for additive it is meant that the molecules of fixative react chemically with the components of the tissue causing the formation of bridges between proteins, while for non-coagulant is meant that the fixative does not replace the water of proteins' hydration, maintaining the relationship with the water molecules.
The fixing inactive lytic enzymes that could digest the tissue and preserves the morphology of the same, avoiding the destruction of antigens. The antigens must be identified in their natural position and made accessible to the primary antibody. Only in this phase we obtain an histological image the most similar to the reality (equivalent image).
After fixing, the samples were processed using an automatic processors, who will carry out the processes of dehydration, infiltration and clearing (*Sakura* Tissue-Tek Xpress®x120 and *Leica* ASP300S).

5.3.1 Dehydration

Within these devices, the sample is first immersed in formalin, to obtain optimal fixation, then thoroughly washed to remove excess fixative (otherwise responsible for artifacts of staining) and subsequently dehydrated by a gradual transition in a scale of alcohols at increasing concentrations (50°, 80°, 95°), until absolute alcohol. The times spent in each alcohol concentration vary on the size of the sample. It is important that the dehydration is complete to allow tissue imbibition with the clearing solvent.

5.3.2 Clearing

After dehydration following clearing. This process needs to replace the alcohol with an organic solvent miscible with paraffin, such as xylene. The clearing needs to modify the properties of a sample placed on a slide so that this can be sufficiently opaque as to let pass the light radiation.

5.3.3 Infiltration

This last step, consists in the immersion of the dehydrated sample in the embedding medium at the liquid state (paraffin at a temperature of 58-60° C) for a time sufficiently long enough to allow penetration into the deepest interstices of the sample.
The embedding materials act as a support for the section, during cutting phase, avoiding sections loss of their consistency.

5.4 EMBEDDING

The tissue sample is then embedded in paraffin.
Paraffin is a waxy semi-solid compound formed by a mixture of aliphatic hydrocarbons.It allows the penetration of the embedding material in the tissue, interrupting quickly and completely the autolytic processes that develop in cells and tissues not more nourished and oxygenated, preserving them from the action of molds and bacteria.
Finally, the paraffin preserves the structural and ultrastructural characteristics of the sample.
Operationally, our samples were placed in a “mold base” (small metal container with appropriate dimensions to the size of the piece), oriented so that the piece must be included in the center to offer the least resistance to the cut, with the cutting surface in contact with the surface of the “mould base”; in this way we got transverse sections of the wall including cardiac entire thickness, from epicardium to endocardium, they were placed in a "mould base.
Next, we have carried to make a pouring of paraffin on the heated material to be included and we have carried with the closing of the "base mould. Every sample embedding report its number histological.
The "mold bases" were subsequently transferred to a cold plate and let it here until of paraffin solidification (5-10 minutes).
After the paraffin is solidified, it is carried to detach the block formed with the sample.

5.5 HISTOLOGICAL SECTION

The section of the samples embedded is one of the most delicate phases of the entire analytical process of preparation of histological samples. The quality of the section is critical for the achievement of a correct diagnosis.
To be observed under the microscope the tissue embedded in paraffin should be cut into thin slices, which allow the light to pass through it and give a good microscopic observation. The microtome is the instrument which cut samples embedded into thin slices.
For our study we used slices with a thickness of 3.5 µm to obtain a single layer of cells per slice, avoiding cellular overlapping that could interfere with the experiments and subsequent readings microscope.
The sections are lay out on microscope slides electrostatically loads that act as support for the staining step allowing, once mounted with coverslips, microscopic evaluation and sample preservation.
The instruments necessary in a cutting station are as follows:
- Rotary Microtome

- Container of cold distilled water for the sections collection
- Thermostatic bath at 45/46 ° C
- Disposable blades
- Freezer at -20° C, or a cold plate for embedding samples cooling
- Brush for sections collection and manipulation
- Slides

The procedure provides various phases:

- Numbering of microscope slides and their choice: the number of acceptance of the patient, with its years of sampling and whether it is standard biological or autopsy sample (or other type), is written with a permanent laboratory pen, on the frosted band of microscope slide.The slides are different depending on the staining type which will be subjected. We used polarized Superfrost® Plus slides for immunofluorescence and immunohistochemical staining.

  - Removal of paraffin excess from histological cassettes from the cutting surface of the embedded sample. The removal of paraffin stops when all the tissue sample emerges on the cutting surface and may be entirely isolated. This step is preferably performed with embedding in paraffin at room temperature.

  - Cooling: the histological cassettes, after removal of paraffin excess, are left to cool in a freezer at -20°C. This phase confers to paraffin a consistency, such as to favor the cutting phase.

- Cutting of the sections: After verification that the blade of the microtome is intact, it sets the slice thickness in relation to the type of staining to be performed (in our case 3.5 µm).

  - Selection of the sections: The sections, with the help of the paintbrush, are placed in cold water that promotes relaxation of any creases. Comparing the section obtained with the section embedded, we have that all tissue is present. The best sections are selected (in absence of creases, scratches or tears) and they are picked up with a cover slip.

  - Expansion of the sections: The sections are transferred in a thermostatic bath containing hot distilled water at about 45/46 °C. At this phase, the paraffin it softens and section is stretched.

  - Pick up of sections: The sections are picked up by paintbrush are placed on microscope slide

  - Draining slides: Slides are placed on a special support that allows the draining of excess water.

- Slides for histochemical and immunohistochemical staining: It is better to use polarized microscope slides and left to dry sections in an oven at 60 °C for about 30-40 minutes, if it appears possible their detachment from the glass during the staining.

5.6 DEWAXING

The histological sections embedded in paraffin ready to be colored must be subjected to dewaxing: tissues are immersed in a sequential scale of solutions that allow to dissolve the paraffin with subsequent hydration of the tissues.

In our case, the slides were immersed in containers with reagents following the order and times indicated as follows:
1. Xylene, 2 changes, each of 3 minutes
2. Xylene-absolute ethanol for 3 minutes
3. Absolute ethanol, 2 changes, each of 3 minutes
4. Ethanol 95%, 1 change from 3 minutes
5. Ethanol 80%, 1 change from 3 minutes
6. Ethanol 70%, 1 change to three minutes
7. Distilled water for at least 2 minutes

5.7 ANTIGEN RETRIEVAL

The identification of antigens in tissues fixed in formalin and embedded in paraffin is limited by the effects of fixatives on the proteins and glycoproteins.
In particular, the fixing can alter the molecular structures and make it less accessible antigens, preventing their localization by primary antibody.
To overcome these limitations, prior to incubation of the histological section with primary antibody, we proceed to the antigen retrieval.
The antigen retrieval chosen for this thesis implies the use of heat and buffered solutions at pH6 or pH9. In particular, the protocols used in this study were the following:

A) citrate buffer, pH 6
Dako EnVision™ FLEX-Target retrieval solution- Low pH (50X)
1. Incubation in citrate buffer, pH6 for 40 minutes at 98 °C
2. cooling for 20 minutes at room temperature (RT)
3. Wash in distilled water for 2 minutes

B) Tris/EDTA buffer, pH 9
Dako EnVision™ FLEX-Target retrieval solution- High pH (50X)
1. Incubation in Tris/EDTA buffer, PH9 for 40 minutes at 98 °C
2. cooling for 20 minutes at room temperature (RT)
3. Wash in distilled water for 2 minutes

5.8 IMMUNOFLUORESCENCE

The immunofluorescence is used to detect simultaneously the presence of multiple cellular antigens, exploiting the specificity of the antigen-antibody reaction and the characteristics of the excitation and emission of different fluorochromes used. After the primary antibody incubation, the method involves incubation with a secondary antibody, conjugated to a fluorochrome, with high binding specificity for the primary antibody.

Microscopic analysis is performed with an epifluorescence microscope equipped with a mercury vapor lamp that produces radiation which, after having been selected by appropriate excitation filters, excite the fluorochromes. Appropriate barrier filters eliminate the emission light of the wavelengths not desired, to detect only the fluorescence light.
The protocol, used by us,includes the following steps:

A) Saturation of the non-specific sites
To reduce the antibody binding to sites other than the antigens of interest, it was used a buffer solution (PBS) containing donkey serum (10% v/v) of the animal species (donkey) in which the secondary antibodies were produced.The sections were then incubated in humid room for 30 minutes at room temperature. Subsequently, the excess serum was eliminated (no wash samples) and was done by incubating the sections with the solution containing the primary antibody.

B) Primary antibodies incubation
Table 5.1 summarizes the optimized conditions to promote specific binding of primary antibodies used in this work.

Antibody	Drug Company	Dilution	Antigen Retrieval pH	Incubation Temperature	Incubation Time
NF-kB Rabbit Polyclonal	Abcam	1:500	pH6	4°C	O.N
NLRP3 Rabbit Monoclonal	Cell Signaling	1:500	pH9	4°C	O.N
α-SA IgM Mouse Monoclonal	Sigma	1:100	pH6	37°C	1 hour
Caspase 1 Mouse Monoclonal	Abcam	1:100	pH9	37°C	2 hours
53BP1 Rabbit Polyclonal	Abcam	1:50	pH6	4°C	O.N

Table 5.1 Primary Antibody, dilution, time and temperature incubation. O.N. = Overnight (12-14 hours).

C) Secondary antibodies incubation

After incubation with the primary antibodies, the excess of unbound antibody was removed by washing the sections with PBS (10 minutes, room temperature).
The secondary antibodies used in immunofluorescence (see Table 5.2) are also diluted in PBS and conjugated to a fluorophore. The secondary antibodies, once diluted, are centrifuged 10 minutes 16,000 rcf at a temperature of 4 °C.
The secondary antibody incubation takes place in the wet room, in an incubator at 37°C for 1 hour, with subsequent washing of the slides in 1X PBS for 5 minutes.

Primary Antibody	Fluorochrome	Drug Company	Excitation Spectrum	Dilution	Temperature and Time Incubation
NF-kB Rabbit Polyclonal	488	Molecular Probe, Invitrogen	488 nm	1:800	37°C, 1 hour

Primary Antibody	Fluorochrome	Drug Company	Excitation Spectrum	Dilution	Temperature and Time Incubation
NLRP3 Rabbit Monoclonal	488	Molecular Probe, Invitrogen	488 nm	1:800	37°C, 1 hour
α-SA IgM Mouse Monoclonal	Cy5	Jackson Immuno Research	650 nm	1:400	37°C, 1 hour
Caspase 1 Mouse Monoclonal	555	Molecular Probe, Invitrogen	555 nm	1:600	37°C, 1 hour
53BP1 Rabbit Polyclonal	488	Molecular Probe, Invitrogen	488 nm	1:800	37°C, 1 hour

Table 5.2 Secondary Antibodies, Dilution, Time and Temperature time

D) Reduction of autofluorescence of the sections of the myocardium

To limit the autofluorescence generated by aldehyde fixatives, after the tissue staining, we colored the section with a solution of Sudan Black (0.1% in 70% ethanol) for 30 minutes at room temperature. Subsequently, the slides are washed first 2 minutes under running water and then in distilled water at room temperature for another 2 minutes.

E) Assembly

To show the nuclei, we used the 4'-6'-diamino-2-phenylindole dihydrochloride (DAPI), a DNA intercalating that emits the blue light spectrum. This dye is added, at the end of the staining, to a aqueous pillar (Vectashield) designed to reduce the decay of fluorescence and photobleaching effect (fluorescence decrease of a sample due to photochemical degradation of the dye). Finally we put a cover glass over the sections and we sealed to reduce the diffusion of oxygen. At this point the slides are ready to be analyzed under the microscope.

5.9 IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry staining differ, from those of immunofluorescence, mainly for the detection system. In particular, dewaxing, antigen retrieval and the primary antibody incubation proceed in a similar way in the two methods.
Our protocol included the following steps:

A) Application of the reagent for blocking endogenous peroxidase

When it is used a detection system that operates in peroxidase, it is necessary remove peroxidase naturally present in tissue to avoid false positives. Specifically, the sections were incubated with a solution of hydrogen peroxide at 3.5% in distilled water, 10 minutes at room temperature, followed by a wash with distilled water (3 minutes, RT) and PBS (10 minutes, RT).

B) Primary antibody incubation

Once dried the slides, the primary antibody was dispensed respecting the concentration and incubation conditions summarized in **Table 5.3**

Antibody	Drug Company	Dilution	Antigen Retrieval pH	Incubation Temperature	Incubation Time
Polyubiquitinated Proteins	Enzo Life Sciences	1:300	pH6	RT	1 hour
LC3	Cell Signaling	1:100	pH6	RT	1 hour
P62/SQSTM1 Mouse Monoclonal	MBL	1:500	pH6	RT	1 hour
Parkin 1	Biorbyt	1:100	pH6	RT	1 hour
4-HNE Rabbit Polyclonal	Antibodies-online	1:500	pH6	RT	1 hour

Table 5.3 Primary Antibody, dilution, time and temperature incubation. R.T. = Room Temperature.

C) Use of the detection system EnVision (Dako Real)

When the primary antibody incubation finished, the slides were dried and was affixed a drop of reagent on each section. The slides were then incubated for 40 minutes at RT. Subsequently it is proceeded to wash the excess reagent with PBS for 15 minutes at RT and has applied the chromogen (DAB) for 1-4 minutes at RT. After this step, the slides were washed for 5 minutes with distilled water and we proceeded to counterstained with Gill's hematoxylin for 1 minute at RT, followed by washing in running water for 2 minutes and in distilled water for 1-2 minutes.

D) Dehydration, clearing, mounting.

At staining end, we proceeded to dehydrate the samples by immersion in ascending scale of the alcohol (80 °to the absolute starting alcohol for at least 2 minutes each alcohol) and clearing by xylene for 2 times for 2 minutes each. Finally, the stained sections were mounted using the medium Eukitt.

5.10 SAMPLING AND ANALYSIS OF IMAGE

A) Images Acquisition and immunofluorescence colors sampling

For each histological sample stained we acquired 7 images of random fields not overexposed using a 63x objective immersion oil (numerical aperture 1.4) of the epifluorescence microscope Leica DMI6000 B (Leica Microsystems).

B) Semi-quantitative evaluation of NF-kB (IF)

To evaluate the positivity for NF-kB in myocytes, it was first created a mask, using the ImageJ program, to exclude interstitial cells from the quantification. Subsequently, it was evaluated: the sectional area occupied by positive staining and the mean fluorescence intensity. We obtained the fluorescence intensity integrated (IFI) by the product of these two parameters,. Similarly, we created a mask such as to exclude the area of the sections occupied by the positivity alpha sarcomeric actin (α-SA), evaluating the positivity to NF-kB no myocytic cells.

C) Semiquantitative evaluation of NLRP3 and Caspase 1 (IF)

To evaluate the positivity for NLRP3, we first proceeded to create a threshold for each image to select the positive area to the marker. Once measured area covered by the positive signal and mean fluorescence intensity (to calculate the IFIs), we created a mask to evaluate co-localization of NLRP3 / Caspase1. Subsequently, on the area NLRP3 positive, we have done a threshold for Caspase1 positivity measuring area and mean fluorescence intensity to calculate the IFI.
The distinction between myocytes and no myocytes was made by alpha sarcomeric actin immunoreactivity, as above.

D) Semiquantitative evaluation of 53BP1 (IF)

For this coloration it has not been possible to use ImageJ, and we preferred to search the fraction of positive cells staining. We therefore carried out a manual counting of nuclei total, positive nuclei, myocytes total nucleated, myocytes nucleated presenting 53BP1 positivity. The α-SA was used to discriminate the best cardiac myocytes in manual counting

E) Calculation of the sectional area and length of myocyte

Using the ImageJ program we performed an evaluation of the myocyte sectional area, manually tracing the cellular perimeter, and a measurement of the myocyte length, tracing a straight segment between the intercalated discs of the two ends of the cardiomyocyte.
The analysis was carried out on samples stained with an antibody anti α-SA, selecting myocytes in perfectly transverse section presenting a central nucleus. For the measurement of the length, we analyzed only the cardiomyocytes showed that one or more nuclei on the periphery of which were obvious accumulations of mitochondria or lipofuscin.

F) Image acquisition and sampling of immunohistochemistry staining

We acquired, for each histological sample stained, 6 non-overlapping images of random fields, using a 20x objective of a Leica microscope DMD108. We maintained constant lamp intensity and acquisition parameters using digital camera.

G) semiquantitative evaluation of 4-HNE (IHC)

Similarly to what was done for the colors of immunofluorescence,we used the program ImageJ to obtain a semiquantitative positivity for the antigen sought. In this specific case, we first proceeded to decompose the individual images in the RGB three channels that compose (red, green and blue). Subsequently, we proceeded to create a threshold for the channel in which the contrast between the gray levels of the positive signal and those of the rest of the section was maximum (in our case, the green channel). We calculated area and mean intensity of the gray level obtaining (by multiplication of these two parameters) the intensity integrated optical (IOD). We also analyzed, making a threshold for the channel in which the contrast between gray levels of the positive and the negative staining area was minimal, the total area covered by the section.We calculated volume fraction (VF) occupied by a positive signal, dividing the area of positivity for the total area of the section.

5.11 STATISTICAL ANALYSIS

For statistical analysis we used the program GraphPad Prism version 4.0.
Data are presented as median and interquartile range, while the differences between the two groups were tested using unpaired t-test samples or test Mann-Witheny, depending on the normal distribution of work.
Finally, the correlations between the parameters tested were analyzed by evaluating the correlation coefficient Spearman's rank.

5.12 WESTERN BLOT ANALYSIS

5.12.1 PROTEIN COLLECTION AND EXTRACTION

The proteins was extracted by biopsies obtained by atria of explanted hearts of 6 patients affected by iDCM and by 6 donors frozen in liquid nitrogen.
The atria, yet frozen, were homogenized in 300 µl of RIPA buffer + inhibitors.
The homogenized tissues were incubated in ice for 1 hour and then centifuged at 13000 rpm for 20 minutes at 4 °C.
The surnatants were collected and the concentration of total proteins contained therein was measured.

5.12.2 MEASUREMENT OF PROTEIN TOTAL CONTENT

Concentration of protein extract was measured using the Bradford assay (Bio-Rad). The Bradford reagents was initially diluted to working dilution with distilled water. BSA standards were prepared, ranging from 0 µg/ml and diluted in 1X Bradford reagent (final volume 1 ml). 2 µl protein lysate was then diluted in 998 µl Bradford reagent, before the lysates and standards absorbance was measured with a spectrophotometer set at 595 nm. The protein concentration of the lysates was calculating using a standard curve generated from the known concentration of the protein standards.

5.12.3 ELECTROPHORESIS AND IMMUNOBLOTTING

For used solutions, see par. XXX 30 µg of total cell lysate was prepared for electrophoresis in sample loading buffer. Samples were boiled at 95°C for 5 minutes and then spun down. Samples were separed by SDS-PAGE gel. The resolving gel is prepared at the right density for the protein of the interest and polymerized between two glass plates in a gel caster. The gel was leveled using saturated isopropanol. After the resolving gel was polymerized, the stacking gel was poured on the first gel, with a comb inserted at the top to create the sample wells. Once polymerized the combs were removed. The gel was put into the Bio-Rad Tetra running tank with 500 ml of 1X running buffer, then the samples and 10 µl of protein ladder were loaded into the wells. The voltage was set a 120V for 1 hour.
Separated proteins were then transferred to nitrocellulose membranes (Schleicher&Schuell). The “sandwich” was prepared with a sponge, 3X filter paper (Bio-Rad), gel, membrane, 3X filter paper and sponge and placed in the cassette. Paper and sponges were soaked in transfer buffer and the tank was filled with running buffer. The voltage was set at 30V for 12 hours at 4°C. The resulting transferred proteins and membrane were then ready to be used in the antibody hybridization method.

5.12.4 ANTIBODY HYBRIDIZATION

Membranes were saturated by incubation at RT for 1 hour with 5% (w/v) BSA in TBS-T and incubated overnight at 4°C with the antibodies listed in Table XX. After three washes with TBS-T, membranes were incubated with secondary antibody coupled to the enzyme peroxidase (HRP) (Sigma-Aldrich). After 1hour of incubation at RT, membranes were washed 3 times with TBS-T and last distilled water, to remove the Tween-20 detergent.

5.12.5 ANALYSIS

The blots were developed using the ECL enhanced chemiluminescence procedure (GE Life Science), which consists of incubating of the membrane with a chemiluminescence solution, resulting in the production of a chemiluminescent signal that is captured onto a film. Equal loading was confirmed by the use of a polyclonal anti-actin antibody (Sigma-Aldrich). The bands were visualized and analyzed using ChemiDoc XRS (Bio-Rad) and associated software. Polyclonal anti-actin antibody (Sigma-Aldrich) was used to normalize the protein content in the total extracts.

ANTIGEN	PRIMARY ANTIBODY	COMPANY	DILUTION, TIME AND TEMPERATURE
LC3B	Rabbit Monoclonal	Cell Signaling	1:1000, O/N, 4°C
BECLIN-1	Rabbit Monoclonal	Cell Signaling	1:1000, O/N, 4°C
ATG3	Rabbit Monoclonal	Cell Signaling	1:1000, O/N, 4°C

ANTIGEN	PRIMARY ANTIBODY	COMPANY	DILUTION, TIME AND TEMPERATURE
ATG7	Rabbit Monoclonal	Cell Signaling	1:1000, O/N, 4°C
ACTIN	Rabbit Monoclonal	Sigma Aldrich	1:1000, O/N, 4°C

Table 5.4: Primary antibodies used for Western Blot

6 - DISCUSSION AND CONCLUSION

The work done in this thesis evaluates the events linking the loss of proteostasis to structural and systematic alterations typically found in patients with idiopathic dilated cardiomyopathy (iDCM).

Precedents studies, made in our laboratory, demonstrate the significant role of senescence in heart failure physiopathology [6] and we wanted to verify, in this work, whether the model we called “senescence vicious circle” may be an important mechanism underlying cardiomyopathy progression to heart failure.

In particular, we have focused our attention on: alteration of autophagic flux alteration, lysosome membrane permeabilization, accumulation of dysfunctional mitochondria with consequent production of lipoperoxides, inflammasome activation, persistence in a state of the DDR, possible activation of pro-inflammatory transcription factors and cardiomyocyte hypertrophy. Furthermore, we evalueted autophagic flux alterations, senescence and lysosome membrane permeabilization also in cardiac stem cells obtained by donors and hearts explanted by patients affected by iDCM.

Experimental evidences suggest that the hearts of patients with iDCM are characterized by arrest of autophagic flow (highlighted by the presence of p62 accumulation, in the presence of similar levels of punctae of LC3) [98]. This is linked to an accumulation of mitochondria marked by Parkin1 protein, suggesting a defect in the removal of dysfunctional mitochondria [99]. In this thesis, we wanted to verify if it was possible to enhance the obtained data showing evidence of mitochondrial dysfunction. For this reason we evaluated, in histological sections of the hearts in the study, the 4-hydroxynonenal. This molecule is a product of lipid peroxidation derived from cardiolipin (a class of phospholipids which is rich in the inner mitochondrial membrane) which, in addition to exercising various biological functions, such as the inhibition of DNA synthesis, enzymes inhibition, triggering of cellular senescence, apoptosis and necrosis activation, also exerts a direct action on the mitochondria [85]. Several studies support the hypothesis that the 4-hydroxynonenal directly damage the mitochondria, causing the dissipation of the proton gradient [85]. The results we have obtained show that, in line with our hypothesis, the explanted hearts of patients with DCM are characterized by accumulation of this molecule, suggesting that mitochondrial dysfunction associated with reduced mitochondrial clearance characterizes this disease.

Experimental evidence has linked mitochondrial dysfunction to inflammasome activation [100] so we wanted to check whether this condition also characterizes the pathophysiology of DCM. We studied NLRP3 receptor which plays a fundamental role in inflammatory response and in hereditary autoinflammatory syndromes (as Cryopyrin-Associated Autoinflammatory Syndromes (CAPS)). This receptor is able to be active in the presence of high levels of ROS, especially in conditions of mitochondrial dysfunction [100]. After activation, NLRP3 undergoes oligomerization followed by association with the adapter ASC protein, which follows the recruitment of procaspasi1. This event leads to activation by cleavage of procaspase, which follows the processing and secretion of cytokines as IL-1β [100]. To assess whether there is evidence of inflammasome activation, in this thesis we have searched and quantified the colocalitation of NLRP3 and caspasi1 positivity, both in myocytes that in interstitial cells. Our data demonstrate that although patients and controls do not differ in NLRP3 levels, myocytes of patients affected by iDCM are characterized by significantly higher levels of caspase 1 colocalizzated in NLRP3 areas positive, suggesting that in these cells, there is the activation dell'inflammasome.

Mithocondrial dysfunction and inflammasome activation are typical elements of cellular senescence [101]. Cellular senescence is a complex cellular response to intrinsix and extrinsic stressor that determines the stop of proliferative cellular activity and causes, in the affected cells, a DNA damage Response (DDR). Senescent cells are characterized by variant phosphorylated histone γH2AX and by colocalization of 53BP1 in this sites (usually telomeric).

To verify if, in hearts of patients affected by iDCM, there is senescent cells accumulation, we evalueted the precence of foci with nuclear positivity for 53BP1 in myocytes and in interstitial cells.

As we have hypothesized, there are most myocytes in DDR state in heart of patients affected by iDCM than in controls. This is consistent with a model in which the altered proteostasis predominantly affects muscle cells as consequence of mutations of proteins of the contractile apparatus.
An important characteristic of cellular senescence is the production and the release, by these cells, of secretome rich of inflammatory cytokines. In this process, NF-κB has a key role because his activation can occur by mechanisms connected together as DDR activation, RIG-1 inflammasome, p38MAPK pathway stress induced, TGFβ and inflammatory cytokines (as IL1β), ceramides and 4-HNE [101]. For this reason, we evalueted if 4-HNE accumulation and DDR increase was associated to activation of this pathway. We observed an increased accumulation of 4-HNE in myocytes and interstitial cells of patients affected by iDCM, as seen with the results previously obtained.
The molecular regulation of cardiac hypertrophy is complex and includes external stimuli that include neurohumoral factors (such as angiotensin II, endothelin and adrenaline), growth factors (such as FGF and TGFβ) and inflammatory cytokines (such as TNF and IL6). Given the potential involvement in the pathogenesis of iDCM, we evalueted hypertrophy parameters as the cross-section area and the length of cardiomyocytes. Hearts of patients affected by iDCM were characterized by increase of both parameters as expected.
We looked for a possible relationship between parameters studied by us. We observed a significant association between inflammasome activation, 53BP1 and NF-κB levels. These results suggest that exist a relationship between parameters analyzed and that the alterations in myocytes may impact the interstitial cells.
We studied if accumulation of protein ubiquitinilated and their receptor $p62^{SQSTM1}$ is associated to autophagic flux alteration. For this reason, we evalueted, by western blotting, proteins involved in autophagy as Beclin, Atg3, Atg7, LC3-II. Beclin has a central role in autophagy. It is expressed in many human and murine tissues and in localized primarly within cytoplasmic structures, including the ER, mitochondria and the perinuclear membrane. Atg3 and Atg 7 are involved in protein ubiquitination anticipating autophagic process. LC3-II is used as indicator of autophagic activity [102]. During autophagy, LC3-I, which is the cytosolic form of LC3, is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine (LC3-II) which is recruited to autophagosomal membranes. Autophagosomal fuses with lysosomes to form autolysosomes which are degraded by lysosomal hydrolases. We noted a significant reduction of all markers in proteins extracted by patients affected by iDCM
Finally we studied senescence, proliferation and lysosomal membrane permeabilization in cardiac stem cells. Our data suggest an increase of p16INK4A and of γH2AX in cells obtained by explanted heart of patients affected by iDCM respect to cells obtained by donors. These data implicate that the cells obtained by iDCM patients are senescent.
We noted also an increase of puntae of galectin-3 in cells obtained by iDCM patients respects to the donors cells. Galectin-3 is a marker for LMP detection so our data indicate

that CSCs by patients affected by iDCM present lysosomal damage and membrane permeabilization.
In conclusion, our results seem to support the hypothesis of the senescence vicious circle, in which proteostasis alteration may cause the arrest of autophagic flux and it results dysfunctional mitochondria accumulation, oxidative stress, accumulation and inflammation activation. Stimulation of cardiomyocytes growth dimensional, mediated by cytokines, can worsen the proteostasis condition already compromised.

7 - REFERENCES

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