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Exploration of altered molecular pathways involved in pathophysiology of LMNA-cardiomyopathy

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Sorbonne Université

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Exploration of molecular pathways involved in pathophysiology of *LMNA*-cardiomyopathy.

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Presented and defended the 7th September 2018

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Abstract

Dilated cardiomyopathy is characterized by enlargement of the left ventricular chamber, compromising cardiac contractility and ultimately resulting in poor left ventricular function. Mutations in *LMNA* gene, encoding nuclear A-type lamins, have been identified in patients presenting dilated cardiomyopathy. This pathology, referred to as *LMNA*-cardiomyopathy, is an anatomic and pathologic condition associated with muscular and electrical dysfunction of the heart, often leading to heart failure-related disability. Although early initiation of treatments may delay progression and prolong the pre-transplantation phase of the disease, more definitive therapies for *LMNA*-cardiomyopathy await better mechanistic understanding of the molecular basis to develop specific treatments. The main aim of my thesis was to decipher molecular pathways implicated in the development of the disease, specially focusing on calcium homeostasis and oxidative stress.

To have a better understanding of the *LMNA*-cardiomyopathy, I used the *Lmna*^{H222P/H222P} mouse model. In the first part of this thesis I showed an increased oxidative stress levels in the hearts of *Lmna*^{H222P/H222P} mice, associated with a decrease of the key cellular antioxidant glutathione. Oral administration of N-acetyl cysteine (NAC), a glutathione precursor, led to a marked improvement of glutathione content, a decrease in oxidative stress markers including protein carbonyls and an improvement of left ventricular structure and function in a model of *LMNA*-cardiomyopathy.

The second part of this thesis aims to investigate the abnormal elevated cardiac expression level of sarcolipin (SLN), which is an inhibitor of the sarco/endoplasmic reticulum (SR) Ca²⁺ ATPase (SERCA) in the hearts of *Lmna*^{H222P/H222P} mice. I studied its implication in the development of the

LMNA-cardiomyopathy inducing an overexpression in cardiac cell lines as well as in wild type mice. Simultaneously, hypothesizing a pathological effect of SLN overexpression, I used RNA interference to inhibit its expression. These findings suggest that sarcolipin is a critical regulator of SERCA in *LMNA*-cardiomyopathy.

Collectively, these results provide molecular insights into *LMNA*-cardiomyopathy and open novel therapeutic avenue for this debilitating cardiac disease.

Résumé

Les cardiomyopathies dilatées sont caractérisées par un affaiblissement du muscle ventriculaire cardiaque gauche (et droit dans les cas les plus sévères) induisant une diminution de la force de contraction. Des mutations du gène *LMNA* codant pour les lamines de type A ont été identifiées comme responsables d'une forme de cardiomyopathie dilatée, i.e. cardiomyopathie-*LMNA*. Elle est caractérisée par des modifications anatomiques associées à une dysfonction musculaire mais aussi électrique du cœur, menant à une incapacité liée à l'insuffisance cardiaque.

Même si une prise en charge précoce peut retarder la progression de la maladie, aucun traitement curatif n'est disponible pour ces patients. Il apparaît donc indispensable d'avoir une meilleure compréhension mécanistique des bases moléculaires de la maladie afin de développer des traitements plus spécifiques.

Le but de mon travail a été de déchiffrer les mécanismes moléculaires impliqués dans la mise en place de la maladie, en se concentrant sur le stress oxydatif et l'homéostasie calcique.

Afin d'avoir une meilleure compréhension de la cardiomyopathie liée à *LMNA*, nous avons utilisé le modèle murin *Lmna*^{H222P/H222P}. D'un côté nous nous sommes intéressés au stress oxydant, nous avons montré une augmentation des niveaux de stress oxydatif dans le cœur des souris porteuses de la mutation *LMNA*, associée à une diminution du glutathion antioxydant cellulaire clé. L'administration orale de N-acétyl cystéine (NAC), précurseur du glutathion, a entraîné une augmentation du taux de glutathion, une diminution des marqueurs de stress oxydatif incluant les protéines carbonyles et les peroxydes lipidiques et une amélioration de la structure et de la fonction ventriculaire gauche dans un modèle de cardiomyopathie-

LMNA.

D'un autre coté nous avons observé un niveau anormalement élevé de l'expression de la sarcolipine (SLN) cardiaque, qui est un inhibiteur de la pompe Ca^{2+} ATPase du réticulum sarcoplasmique (SERCA). J'ai donc étudié son implication dans l'instauration de la cardiomyopathie-*LMNA* en induisant sa surexpression *in vitro* et *in vivo*. Simultanément, dans l'hypothèse d'un rôle délétère de la surexpression de SLN, nous avons inhibé l'expression de la protéine en utilisant un ARN d'interférence délivré par AAV9. De plus, afin de disposer d'un modèle *in vitro*, nous avons développé deux lignées cellulaires cardiaques sur-exprimant la SLN. Nos résultats suggèrent que la SLN pourrait être un régulateur essentiel de la pompe SERCA dans le muscle cardiaque dans le cadre de la cardiomyopathie-*LMNA*.

Collectivement, nos résultats fournissent des possibles pistes thérapeutiques pour la cardiomyopathie-*LMNA*.

Abbreviations

A.

ACE: angiotensin converting enzyme
AAV: adeno associated virus
ADLD: Autosomal Dominant Leukodystrophy.
AKT or PKB: protein kinase B
AP: action potential
ARVC: arrhythmogenic right ventricular cardiomyopathy
AV: atrio-ventricular
aWRN: atypical Werner syndrome

B.

BAF: Barrier-to-Autointegration Factor
BMP: bone morphogenic protein
Bpm: beats per minute
BSA: bovine serum albumin
BW: body weight

C.

Ca²⁺: calcium
CAM: calmodulin
CaMK_{II} or CaMK₂: Ca²⁺/calmodulin-dependent protein kinase II
CAT: catalase
CICR: calcium induce calcium release
CMT: Charcot-Marie-Tooth disease
CSQ: calsequestrin

D.

DCM: dilated cardiomyopathy
DMD: Duchenne muscular dystrophy
DNA: deoxyribonucleic acid

E.

E-C coupling: excitation contraction coupling
EDMD: Emery-Dreifuss muscular dystrophy
ERK_{1/2}: extracellular signal-related kinases _{1/2}
ESCs: embryonic stem cells

F.

FBS: fetal bovine serum
FPLD: familial lipodystrophy of Dunnigan type
FS: fractional shortening

G.

GFP: green fluorescent protein

GPX: glutathione peroxidase

GSH: glutathione

H.

H₂O₂: hydrogen peroxidase

HCM: hypertrophic cardiomyopathy

HF: heart failure

HGPS: Hutchinson-Gilford Progeria Syndrome

I.

Ig: immunoglobulin

INM: inner nuclear membrane

IVS: inter ventricular septum

J.

JNK : c-Jun N-terminal kinase

K.

KASH: Klarsicht, ANC-1, Syne Homology

KDa: Kilodaltons

KI: knock-in

KO: knock-out

L.

LADs: lamina-associated domains

LBR: Lamin B receptor

L-CMD: *LMNA*-related congenital muscular dystrophy

LGMD: Limb-Girdle Muscular Dystrophy

LINC: linker of nucleoskeleton and cytoskeleton.

LMNA-DCM: *LMNA*-cardiomyopathy

L-type channels: long-lasting type channels

LV: left ventricle

LVEDD: left ventricular end diastolic diameter

LVESD: left ventricular end systolic diameter

LVM: left ventricular mass

LVFS: left ventricular fractional shortening

M.

MAPK: mitogen-activated protein kinase

Mg²⁺: magnesium

MOI: multiplicity of infection

mTOR: mammalian target of rapamycin

N.

NAC: N-acetyl-cysteine

Na⁺: sodium

NADPH: Nicotinamide-Adenine-Dinucleotide-Phosphate

NCX: sodium-calcium exchanger

NE: nuclear envelope

NETs: nuclear envelope transmembrane proteins

NL: nuclear lamina

NOX: NADPH oxidase

NPCs: nuclear pore complexes

O.

O²⁻: superoxide anion

OH: hydroxyl radical

ONM: outer nuclear membrane

P.

PCR: polymerase chain reaction

PIP2: phosphatidyl-inositol 4,5 biphosphate

PKA: protein kinase A

PKC: protein kinase C

PKG: protein kinase G

PLN: phospholamban

PW: left ventricular posterior wall

R.

RCM: restrictive cardiomyopathy

RNA: ribonucleic acid

ROS: reactive oxygen species

RyR: Ryanodine receptor

S.

SA node: Sinus-atrial node

SCD: sudden cardiac death

SDS: sodium dodecyl sulphate

SERCA: sarcoplasmic reticulum calcium ATPase

Sh: short hairpin

SIRT6: sirtuin 6

SLN: sarcolipin

SOD: superoxide dismutase

SR: sarcoplasmic reticulum

SUN: Sad1 and UNC-84

T.

TGF- β : transforming growth factor- β

X.

XPG: xeroderma pigmentosum group A.

W.

WT: wild type

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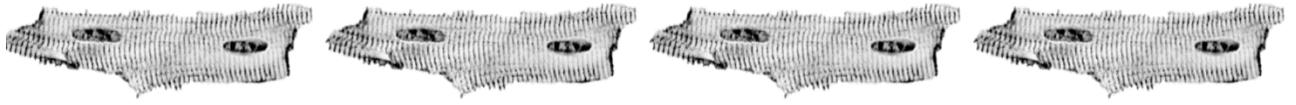
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Preamble

Dilated cardiomyopathy caused by *LMNA* mutations is an aggressive disease of the heart, with no curative treatment to date. The two aims of my thesis were 1) to assess the oxidative stress and 2) to understand the impact of calcium regulators imbalance in dilated cardiomyopathy caused by *LMNA* mutations.

This doctoral thesis is divided into three parts:

- A general introduction to the scientific topics addressed in the dissertation,
- Two peer-reviewed articles, which document and discuss my scientific work. One of the peer-reviewed article focuses on the role of oxidative stress and the second one focus on the role of sarcolipin (SERCA inhibitor) overexpression in the pathology.
- A concluding chapter summarizing the principal outcomes of my work.



Introduction

Chapter 1: Introduction

1. Cardiac muscle

1.1 The heart and its functions

The heart is the first organ to form during development. It is responsible for pumping the blood and therefore, transporting nutrients, oxygen and hormones to the body. The heart is divided into four chambers: two atria (upper chambers) and two ventricles (lower chambers) that ensure the blood pumping (**Figure 1A-C**). To assure normal circulation, the heart presents different valves in each chamber, which prevent the backward blood flow. These valves act as one-way inlets of blood on one side of a ventricle and one-way outlets of blood on the other side of a ventricle. Each valve has three flaps (i.e., tricuspid), except the mitral valve, which has two flaps. The four valves are: 1) pulmonary valve located between the right ventricle and the pulmonary artery and allows the blood flow from the heart to the lungs, 2) tricuspid valve between the right chambers, 3) mitral valve between the left chambers, and 4) aortic valve between the left ventricle and the aorta, which carry blood from the heart to the body (**Figure 1A-C**).

1.1.1 Cardiac contraction

The cardiac contraction can be separated in systole and diastole. Systole is the period when ventricles contract, allowing pressure to rise. It begins when ventricles contract and ends when ejection stops. Diastole begins when ejection ceases as ventricles relax. Ventricular contraction causes ejection of blood through the pulmonary valve, into the pulmonary arteries. This allows blood to get oxygenated and to come back to the left atrium via the pulmonary veins. During systole, arterial blood reaches its peak (systolic blood pressure)

normally between 90 to 120 mmHg. Then the atrium contracts and blood enters the left ventricle through the mitral valve. When left ventricle is loaded with blood, the mitral valve closes and the contraction of the ventricle allows the blood flow through the aortic valve to the aorta. Blood comes back to the heart through the superior and inferior vena cava into the right atrium. When the right atrium contracts, the blood passes through the tricuspid valve into the relaxed right ventricle (**Figure 1D**)(Phibbs, 2007).

1.1.2 Conduction system of the heart

Effective functioning of the heart requires that all parts of the separate chambers contract in a synchronic movement. This is achieved by an efficient conduction system (**Figure 1E**) composed of: 1) sinoatrial node (SA node) located in the right atrium at the entry point of the superior vena cava. Being the natural pacemaker of the heart, the SA node initiates heartbeats and determines heart rate. Electrical impulses from the SA node spread throughout both atria and stimulate them to contract; 2) atrioventricular node (AV node) located at the opposite side of the right atrium compared to the SA node (near the AV valve). It serves as an electrical gate to the ventricles. It delays the passage of the electrical impulses to the ventricles. This delay allows the total ejection of the blood from the atria into the ventricles before they contract. The AV node receives signals from the SA node and sends them to the bundle of His; 3) bundle of His located in the septum is divided into right and left branches, which conduct the impulses through the apex. The electrical impulse is then passed into Purkinje fibres turning upwards and spreading the signal through the ventricular myocardium.

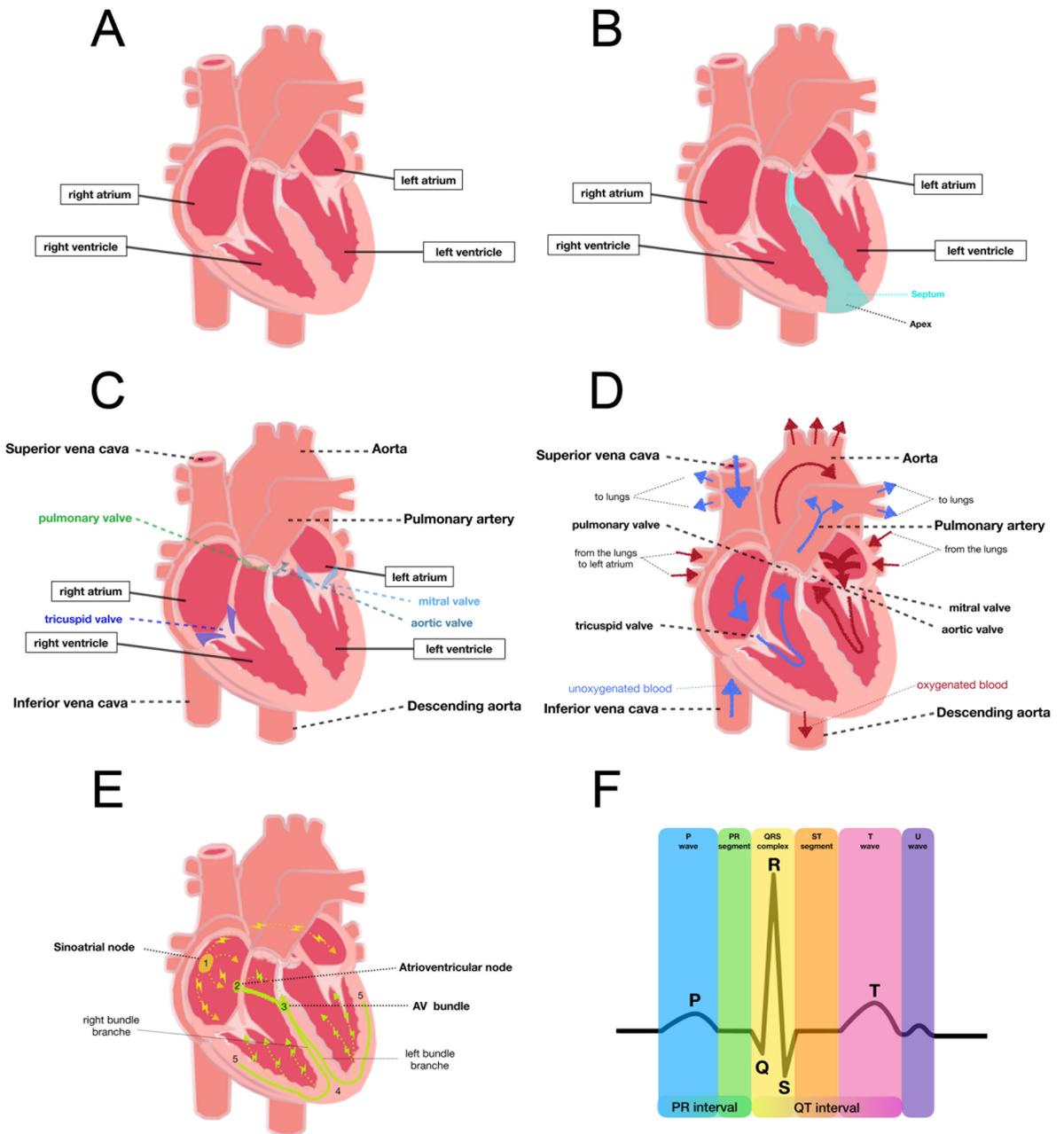


Figure 1: Heart anatomy and physiology A. Cardiac chambers. B. Cardiac anatomy. C. Cardiac valves. D. Cardiac circulation. E. Conduction system F. Electrocardiogram.

Electrocardiography allows recording cardiac electrical activity using electrodes placed on the skin. It comprises several waves and intervals: 1) the P wave corresponds to atrial depolarization; 2) the PR interval is the time between the first deflection of the P wave and the first deflection of the QRS complex; 3) the QRS wave complex is composed of: the Q wave that corresponds to depolarization of the inter-ventricular septum, the R wave that reflects the depolarization of the main mass of the ventricles and the S wave that signs the final depolarization of the ventricles at the base of the heart; 4) the ST segment is the time between the end of the QRS complex and the start of the T wave. It reflects the period of zero potential between ventricular depolarization and repolarization; 5) the T wave represents ventricular repolarization and 6) the U wave is only visible when the heart rate falls below 65 bpm. The source of it is unknown, three theories regarding its origin are: delayed repolarisation of Purkinje fibres, prolonged repolarisation of mid-myocardial and after-potentials resulting from mechanical forces in the ventricular wall (**Figure 1F**)(Ashley and Niebauer, 2004; U Wave basic patterns - LITFL ECG Library).

1.2 The cardiac muscle cell

The mammalian heart is composed of several cell types: cardiomyocytes, fibroblasts, endothelial cells and perivascular cells (Zhou and Pu, 2016). Contracting more than 3 billions times during average lifespan in human, cardiomyocytes are the most physically energetic cells in the body, and therefore contain an important number of mitochondria. Cardiomyocytes are highly specialised cells to assure their functions: 1) the plasma membrane (known as sarcolemma) has invaginations called T-tubules for the fast propagation of action potential; 2) the cytoplasm (known as sarcoplasm)

contains a high number of mitochondria; 3) a specialised endoplasmic reticulum (known as sarcoplasmic reticulum) that contains a high concentration of calcium; 4) and the contractile apparatus composed of myofibers consisting of sarcomeres (**Figure 2A**).

1.2.1 General overview of cardiomyocytes components

A. Sarcolemma

Going from the outside to the inside of the cell, the first specialised structure is the plasma membrane: the sarcolemma. It serves as a barrier for diffusion and contains membrane proteins, including receptors and ion channels (**Figure 2B**). The sarcolemma forms two specialised regions: the intercalated disks and the transverse tubules. The intercalated disks are structures that allow communication and anchoring between adjacent cardiomyocytes. Intercalated disks are composed of several types of junctions: gap junctions, adherence junctions and desmosomes. Transverse tubules (also called T-tubules) are invaginations of the sarcolemma that allow the propagation of a fast action potential through the membrane deep into the cytoplasm and contain high density of L-type channels. These invaginations allow the rapprochement of L-type channels and the sarcoplasmic reticulum, more especially to the Ryanodine receptors (RyR). Other calcium channels implicated in the excitation contraction coupling (E-C coupling) are also located in this membrane as the sodium-calcium exchanger (NCX) (Walker and Spinale, 1999).

Calcium channels located in the sarcolemma:

- **L-type channels:** also known as dihydropyridine receptors, are voltage dependent calcium (Ca^{2+}) channels composed of different subunits. They are heterotetrameric polypeptides complexes containing α_1 , α_2/δ , β subunits. α_1 , is the main functional component, which contains the voltage sensor and two of its transmembrane segments form the pore of the canal. α_2/δ , β are auxiliary subunits implicated in modifying the gating properties of the channel, as well as the channel expression level. The combination of all subunits allows the depolarization-induced calcium influx into the cytosol, which is essential for the calcium-induced-calcium release (CICR) process. The close proximity of L-type channels and RyR2 allows the calcium influx across the plasma membrane to trigger the massive release of calcium from the SR stores through RyR2 opening. L-type channels are mainly localised in the T-tubules facing the SR assuring the fast passage of the Ca^{2+} to the cytosol (Bodi et al., 2005; Kamp and Hell, 2000).
- **NCX:** Sodium-calcium exchanger channel is a key actor of cardiac relaxation, located at the sarcolemma, including at the T-tubules. It is responsible of the extrusion of the cytosolic Ca^{2+} via a transport of three sodium ions (Na^+) in exchange for one Ca^{2+} . Under physiological conditions, NCX removes the same amount of Ca^{2+} that entered the cell through L-type channels in order to maintain cellular Ca^{2+} balance. In the heart, the isoform 1 is mostly expressed. NCX activity can be regulated by Na^+ , Ca^{2+} , protons, phosphatidyl-inositol 4,5 biphosphate (PIP2) in the membrane, protein kinase A (PKA) and exogenous agents. (Ottolia et al., 2013; Schulze et al., 2003).

B. Sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is a specialised endoplasmic reticulum that serves as a source and internal storage of cytosolic Ca^{2+} required for excitation-contraction coupling. SR contains proteins that are essential for calcium homeostasis as the ryanodine receptor (RyR), the sarcoplasmic reticulum calcium ATPase (SERCA) and his two regulators phospholamban (PLN) and sarcolipin (SLN).

- **RyR:** Ryanodine receptor mediates intracellular Ca^{2+} release during contraction, being a key actor of calcium induce-calcium release (CICR). In the heart, the most expressed isoforms is RyR2, but other isoforms have been detected. It is a homotetramer regulated directly or indirectly by L-type channels, Ca^{2+} , magnesium (Mg^{2+}), protein kinase A (PKA), FK506 binding proteins (FKBP12 and 12.6), calmodulin (CaM), Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), calsequestrin (CSQ), triadin and junctin (Lanner et al., 2010).
- **SERCA:** Sarco-endoplasmic calcium ATPase is responsible for muscle relaxation due to Ca^{2+} re-uptake. In heart, the most expressed isoform is the SERCA2a. Its ATPase activity assures the transport of two Ca^{2+} ions per ATP molecule. It is composed by a catalytic subunit with usually ten transmembrane spans and four intracellular loops in which is located the ATP and the phosphorylation sites (Sweedner and Donnet, 2001). The activity of this pump is regulated by two small proteins: phospholamban (PLN) and sarcolipin (SLN) (Periasamy and Kalyanasundaram, 2007). PLN and SLN have a similar structure and roles but their inhibitory procedures are different.
- ◇ **PLN:** Phospholamban is a 52-amino-acids protein (6 KDa) that associates into pentamers to interact and inhibit SERCA by lowering

its Ca^{2+} affinity. It can exclusively bind to SERCA when there is a low Ca^{2+} concentration. Its activity is controlled by the phosphorylation of its Serine₁₆ residue via PKA or its Threonine₁₇ residue via CaMKII and calcium concentration. (Asahi et al., 2003a). Upon phosphorylation, PLN is released from SERCA, allowing calcium to be pumped back into the SR thus leading to a decrease in cytoplasmic calcium.

- ◇ **SLN:** Sarcolipin is a 31-amino-acids protein that interacts and inhibits SERCA by lowering its V_{max} . SLN can bind to SERCA at any cytosol calcium concentration. It has been shown to be an important mediator of muscle thermogenesis due to its interaction with the SR ATPase pump allowing the ATP hydrolysis but decreasing the Ca^{2+} transport (Bal et al., 2012; Pant et al., 2016).

C. Contractile apparatus

The contractile apparatus formed by sarcomeres is a highly organised array of myofilament proteins composed primarily of thick myosin, thin actin filaments and tropomyosin-troponin complex. The overlapping of these proteins gives to the cardiac muscle its striated appearance. Sarcomeric myosins convert the chemical energy of ATP into mechanical energy upon binding to actin thin filaments and promote sarcomere shortening (**Figure 2D**).

1.2.2 Cardiomyocyte contraction

The contractility of cardiomyocyte is under the control of a spatially defined meshwork of ion channels and exchangers that control Ca^{2+} entry. The cardiac contraction is initiated by electrical impulse known as action potential (AP) coming from the SA node inducing a depolarization of the sarcolemma. The AP can be separated in five different phases: 1) phase 0 depolarization; 2) phase 1 early repolarization; 3) phase 2 plateau phase; 4) phase 3 repolarization; and 5) phase 4 resting phase, described in **Figure 2C**. The depolarization propagates throughout the cardiomyocyte reaching the T-tubules, inducing the opening of the L-type channel and calcium conductance. The influx of calcium passing through L-type channels that reaches the sarcoplasm activates the Ryanodine channels. The activation results in an immediate release of large amounts of Ca^{2+} from the sarcoplasm (Fabiato, 1983). The increase of cytosolic Ca^{2+} concentration allows the binding of Ca^{2+} to troponin C, resulting in a shift of troponin I affinity from the actin filament to troponin C. This induces a shift of the troponin-tropomyosin complex away from the actin-myosin binding site. The shift allows myosin to swing towards the thin filament (consuming ATP). This conformational change generates a force moving the thin filament relative to the thick filament. This cycle will continue until the Ca^{2+} is removed from the cytoplasm by active, energy-dependent means (by SERCA activity) or by exhaustion of ATP stores (Walker and Spinale, 1999).

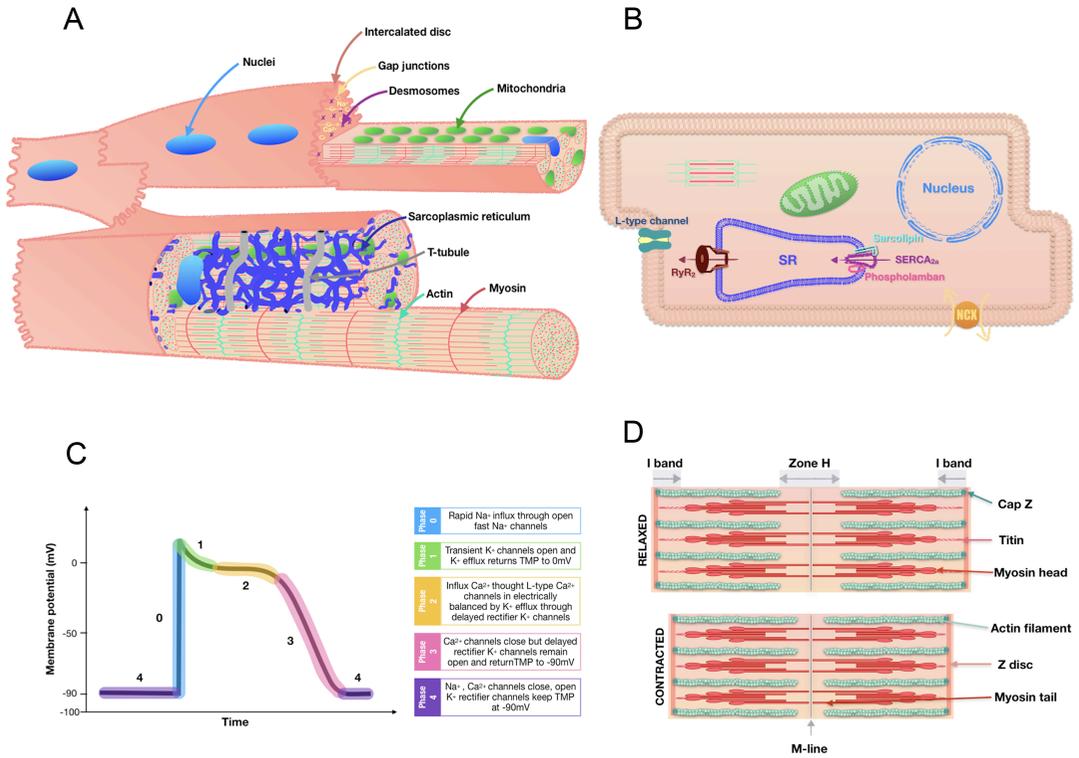


Figure 2: Cardiac cellular structure and components. A Cardiac muscle fiber. **B.** Schematic representation of a cardiomyocyte, and its calcium channels. **C.** Action potential. **D.** Sarcomere composition

2. Cardiomyopathies

2.1 Definition

Cardiomyopathies are a group of cardiac disorders characterized by a cardiomyocytes dysfunction and tissue-wide remodelling of the myocardium leading to functional decline. The prevalence arises with the age due to the age-related changes that can induce pressure overload. Distinct techniques as echocardiography¹, electrocardiography² and cardiac magnetic resonance imaging³ allow the characterisation of these pathologies. Features like severity, distribution, extent of myocardial hypertrophy, thickening of valves, ventricular dilatation, myocardial fibrosis and detection of infiltrates can be determined using these techniques and are essential for diagnosis (Elliott et al., 2008; McKenna et al., 2017).

2.2 Classification

The classification of cardiomyopathies has always been a source of debate. The first classification based on structural and functional changes was proposed by Goodwin in 1961, including congestive cardiomyopathy, hypertrophic cardiomyopathy and constrictive cardiomyopathy (Goodwin et al., 1961). Since then, worldwide cardiac societies propose regularly classifications for these pathologies. Currently four subgroups of cardiomyopathies are defined: 1) hypertrophic cardiomyopathy (HCM), 2)

¹ **Echocardiography:** non-invasive ultrasound technique allowing the visualisation of the pumping function, the chamber size and the velocity of blood flow of the heart.

² **Electrocardiography:** non-invasive technique recording the electrical activity of the heart by electrodes attached to the surface of the skin.

³ **Magnetic resonance imaging:** non-invasive medical imaging technology for assessment of the function and structure of the cardiovascular system.

restrictive cardiomyopathy (RCM), 3) arrhythmogenic right ventricular cardiomyopathy (ARVC) and 4) dilated cardiomyopathy (DCM). Each phenotype is subclassified depending on the causes of the pathology (Figure 3) (Charron et al.).

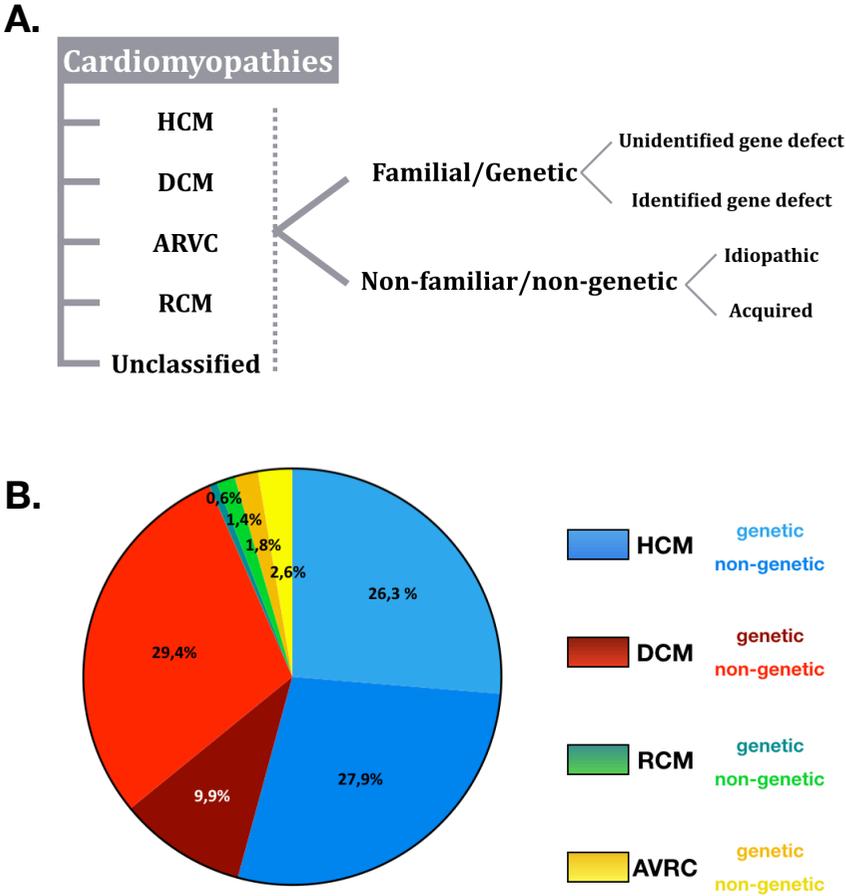


Figure 3: Cardiomyopathies: actual classification and prevalence. A. Cardiomyopathies classification adapted from Elliot *et al* 2008. B. Representation of the European cardiomyopathy prevalence in 2018 (3208 patients from EURObventional research program).

2.2.1 Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is characterized by an increased left ventricular (LV) wall thickness that is not solely explained by abnormal loading conditions. The LV wall thickness is $\geq 13\text{mm}$ and presents a normal or increased ejection fraction. The cardiomyocytes are hypertrophied, disorganised and separated by areas of interstitial fibrosis. HCM is a pathology affecting both children and adults and is the major cause of sudden death in teenagers and elite athletes. HCM is the most common cardiomyopathy and can be inherited or acquired. It is commonly acquired in an autosomal dominant pattern of inheritance but other patterns have been described. The mutations of different genes lead to HCM but genes encoding for proteins of the sarcomere as myosin-binding protein C, myosin heavy chain, cardiac troponin I, α -tropomyosin, cardiac α -actin, myosin light chains 2 and 3 and cysteine and glycine-rich protein 3 are responsible of most reported cases (Marian and Braunwald, 2017; 2014).

2.2.2 Restrictive cardiomyopathy

Restrictive cardiomyopathy (RCM) is characterized by an increased myocardial stiffness that leads to impaired ventricular filling. RCM affects either one or both ventricles but their size is not affected until late stages of the pathology. It is frequently accompanied of arrhythmias and conduction disturbances. RCM is the least common cardiomyopathy and it can be inherited in an autosomal dominant manner or acquired. Most cases of RCM are acquired, however some mutations are recurrent affecting the genes encoding for Troponin T and I, α -actin and β -myosin heavy chain (Mughtar et al., 2017).

2.2.3 Arrhythmogenic right ventricular cardiomyopathy

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is characterized by fibro-fatty replacement of the right ventricular myocardium leading to paroxysmal ventricular arrhythmias and a predisposition to sudden cardiac death (SCD)(2nd most common cause of SCD in children, young adults and athletes). ARVC manifests with electrocardiographic abnormalities as syncope or ventricular arrhythmias. Sustained ventricular tachycardia and/or ventricular arrhythmia lead to SCD. In a cellular level cardiomyocytes are progressively necrotic and frequently accompanied by inflammation, fatty infiltration and replacement of cardiomyocytes. ARVC it is typically inherited in an autosomal dominant manner, with variable penetrance and expression. Most cases of ARVC are due to mutations on the genes encoding for the proteins located in the desmosome (junction plakoglobin, desmoplakin, phakophilin-2, desmoglein-2, desmocollin-2). However mutations in non-desmosomal genes such as genes encoding for cardiac ryanodine receptor 2, transforming growth factor β -3, the nuclear transmembrane protein 43 and desmin had also be reported to induce ARVC. (Basso et al., 2018; Corrado et al., 2017; Mestroni and Sbaizero, 2018; Priori et al., 2015)

2.2.4 Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is characterized by an enlargement and an impaired systolic function of left or both ventricles. DCM can include atrial and/or ventricular arrhythmias and conduction defects. It is a major risk factor for developing heart failure (HF) and sudden death can occur at any stage of the disease. DCM is the second most common cardiomyopathy (**Figure 3B**). DCM can be attributed to genetic alterations, main genetic causes responsible of the pathology are summarised in **table 1**. The majority of the

inherited DCM is autosomal dominant. Inherited DCM is genetically heterogeneous due to mutations of proteins forming the sarcomeres, the Z disc, the dystrophin complex, the cytoskeleton, the desmosomes, the sarcoplasmic reticulum and cytoplasm, the ion channels, the mitochondria, the extracellular matrix, the lysosomes, the nucleus and the nuclear envelope. Within all familial forms of DCM, *LMNA*-cardiomyopathy is the second most common pathology (Hershberger et al., 2010) and is the focus of my work. Patients with *LMNA*-cardiomyopathy have been reported to have a worse clinical prognosis than DCM patients carrying different pathologic DCM-associated gene mutations (McNally and Mestroni, 2017; Pinto et al., 2016; Wang et al., 2017).

Medical terms	
Ejection fraction	Percentage of blood ejected out from the heart chamber with each beat.
Bradyarrhythmia	Reduced heart rate: Sinus rhythm with a resting heart rate of 60 beats per minute or less.
Atrioventricular block	Interruption or delay of electrical conduction from the atria to the ventricles due to conduction system abnormalities in the AV node or the His-Purkinje system.
Atrial arrhythmia	Abnormal heart rate (60-100 bpm): being too fast (tachyarrhythmia) or too slow (bradyarrhythmia).
Atrial fibrillation	Irregular and uncoordinated contraction. Absence of P waves with an atrial rate of 350-600 beats/min, irregular ventricular rhythm, ventricular rate 100-180 beats/min
Conduction system disease	Disruption of the electrical impulses of the heart. It can be due to a defect in the sinoatrial node, intermodal tract, atrioventricular node right or left bundle branches or the bachman's bundle. The three main types of conduction system disease are the heart block, long QT syndrome and the bundle branch block.

Genetic dilated cardiomyopathies		
Affected organ(s)	Disease/affected gene	
Predominant cardiac phenotype	- Titin (<i>TTN</i>)	familial DCM % ~ 20-25 %
	- Lamin A/C (<i>LMNA</i>)	~ 6 %
	- Myosin heavy chain (<i>MYH7</i>)	~ 4 %
	- Troponin T (<i>TNNT2</i>)	~ 2 %
	- Myosin-binding protein C (<i>MyBPC3</i>)	~ 2 %
	- RNA-binding modif-20 (<i>RBM20</i>)	~ 2 %
	- Myopalladin (<i>MYPN</i>)	~ 2 %
	- Sodium channel alpha unit (<i>SCN5A</i>)	~ 2 %
	- BaCl2-associated athanogene 3 (<i>BAG3</i>)	~ 2 %
	- Phospholamban (<i>PLN</i>)	~ 1 %
Neuromuscular disorders	- Duchenne muscular dystrophy (<i>DMD</i>)	
	- Becker muscular dystrophy (<i>DMD</i>)	
	- Myotonic dystrophy or Steinert myopathy (<i>DMPK</i>)	
Syndromic diseases	- Mitochondrial diseases (mtDNA or nuclear genes)	
	- Tafazin (<i>TAZ</i>)	
Acquired dilated cardiomyopathies		
Group	Causing agent	
Drugs	- Antineoplastic drugs	
	- Psychiatric drugs	
	- Other drugs	
Toxic and overload	- Ethanol	
	- Cocaine, amphetamines, ecstasy	
	- Other overload	
	- Iron overload	
Nutritional deficiency	- Selenium deficiency	
	- Thiamine deficiency	
	- Zinc and copper deficiency	
	- Carnitine deficiency	
Electrolyte disturbance	- Hypokalaemia, hypophosphatemia	
Endocrinology	- Hypo- and hyper-thyroidism	
	- Cushing/Addison disease	
	- Phaecromocytoma	
	- Acromegaly	
	- Diabetes mellitus	
Infection	- Viral	
	- Bacterial	
	- Mycobacterial	
	- Fungal	
	- Parasitic	
Auto-immune diseases	Organ specific	
	- Giant-cell myocarditis	
	- Inflammatory DCM (non-infectious myocarditis)	
	Not organ specific	
	- Polymyositis/dermatomyositis	
Peripartum		

Table 1: Aetiology of dilated cardiomyopathies (adapted from Pinto *et al* 2016).

3. *LMNA* pathologies

3.1 *LMNA*-cardiomyopathy

LMNA-cardiomyopathy is characterized by a left ventricular dilatation and/or reduced systolic function (left ventricular ejection fraction <50%). It is frequently accompanied by significant conduction system disease and arrhythmias. The conduction system disease commonly precedes development of DCM. It is characterized by: 1) sinus bradycardias, 2) sinus node arrests with junctional rhythms, 3) a high frequency of atrioventricular blocks, 4) atrial fibrillation and 5) ventricular arrhythmias. Sudden cardiac death (SCD) due to the ventricular arrhythmias occurs frequently, often before the development of DCM (30% of the cases). First signs of *LMNA*-cardiomyopathy usually appear at mid-age (20's 30's) usually with conduction system disease or atrial arrhythmia but some patients are asymptomatic. A gradual worsening of the pathology occurs leading to a full penetrance at the age of 60-70 years (Hasselberg et al., 2018; Hershberger and Morales, 2016; Hershberger et al., 2010; Wang et al., 2017).

No curative treatment is currently available for *LMNA*-cardiomyopathy patients, but research on mouse models have identified different potential therapeutic targets (Worman, 2018), some being currently tested in clinics (NCT02057341). Current clinical management strategies for *LMNA*-cardiomyopathy patients are the ones used for patients with other cardiomyopathies. Individual symptoms of the pathology are treated to maintain the cardiac function compiled in **table 2**. The management of *LMNA*-cardiomyopathy is focused on treatment of conduction system disease, arrhythmias, and DCM (Hershberger and Morales, 2016), the patients commonly require implantable cardiac defibrillators in order to prevent SCD (Meune et al., 2006).

Therapies	
Manifestation	Treatment
Conduction system disease and arrhythmia	
Chronic atrial fibrillation	Anticoagulants combined with agents for ventricular rate control.
Supraventricular arrhythmia	Pharmacological agents and sometimes atrial or atrioventricular node ablations.
Symptomatic bradycardias or asymptomatic but with heart blocks	Implantable electronic pacemaker or implantable cardiac defibrillator (more suitable for <i>LMNA</i> mutation carrier patients due to the high risk of SCD).
Symptomatic ventricular arrhythmias, ventricular tachycardia, ventricular fibrillation, and resuscitated sudden cardiac death	Implantable cardiac defibrillator accompanied of pharmacological treatment.
Dilated cardiomyopathy	
Symptomatic dilatation	Pharmacological agents (ACE inhibitors, beta blockers, diuretics...).
Dilatation with deterioration of LV function (EF <30%)	Pharmacological agents + anticoagulation to prevent the development of left ventricular mural thrombus and embolic events including stroke.
Progressive DCM, advancing heart failure	Heart transplantation.

Table 2: Current treatments for *LMNA*-cardiomyopathy.

In addition to *LMNA*-cardiomyopathy, *LMNA* mutations have been described in patients with severe forms of arrhythmogenic right ventricular cardiomyopathy (Quarta et al., 2012), restrictive cardiomyopathy (Paller et al., 2018) and in patients with an increased risk of thromboembolic complications (van Rijsingen et al., 2013).

LMNA mutations are not only responsible for cardiac diseases but also lead to a broad spectrum of pathologies called laminopathies (described in 3.2.). It is important to note that the cardiac involvement in laminopathies is not exclusive to *LMNA*-cardiomyopathy. It is also present in patients with Emery-Dreifuss muscular dystrophy, Limb girdle muscular dystrophy,

Progeria syndromes and lipodystrophies. The main characteristics of the cardiac involvement in these pathologies are summarised in **table 3**.

Laminopathies with heart involvement		
Pathology	Cardiac phenotype	Ref
Dilated cardiomyopathy with conduction defects	Left ventricle dilatation, systolic dysfunction, atrioventricular conduction block, arrhythmia, congestive heart failure	(Taylor et al., 2003)
Emery Dreifuss muscular dystrophy	Atrioventricular conduction block, arrhythmia, systolic dysfunction, congestive heart failure	(Zhang et al., 2015)
Limb girdle muscular dystrophy	Atrioventricular block, progressive left ventricle dysfunction, arrhythmia	(Chang et al., 2010)
Variant progeroid syndrome with right ventricular cardiomyopathy	Right atrium and ventricle dilatation, tricuspid valve dilatation	(Alastalo et al., 2015)
Atypical progeroid syndrome with cardiomyopathy	Right ventricle dilatation, arrhythmia, tricuspid valve regurgitation	(Guo et al., 2016)
Familial partial lipodystrophy of Dunningan type 2	Left ventricle dilatation, systolic dysfunction, atrioventricular block, complete left bundle branch block	(Andre et al., 2015)
Lipodystrophy with hypertrophic cardiomyopathy	Left ventricle hypertrophy, aortic valve calcification, stenosis and regurgitation	(Duparc et al., 2009)
Charcot-Marie-Tooth type 2 axonal neuropathy	Left ventricle dilatation, systolic dysfunction,	(Duparc et al., 2009)
Severe metabolic syndrome caused by a <i>ZMPSTE24</i> mutation	Left ventricle dilatation, systolic dysfunction, ventricular extra systole	(Galant et al., 2016)

Table 3: Cardiac involvement in laminopathies.

3.2 Laminopathies

Laminopathies are a wide spectrum of pathologies caused by mutations in genes encoding for nuclear lamins and associated proteins. They are commonly divided in 4 groups depending on the affected tissue: 1) striated muscle, 2) adipose tissue, 3) nervous system and 4) accelerated aging syndromes.

During the 90's the first mutations of the genes responsible of laminopathies were identified. In 1994 Bione *et al.* reported a mutation in *EMD* gene leading to X-linked Emery–Dreifuss muscular dystrophy (EDMD), not long after Bonne *et al.* reported the first *LMNA* mutation leading to autosomal dominant forms of EDMD (Bione et al., 1994; Bonne et al., 1999). Since then, more than 460 mutations in *LMNA* gene have been reported (<http://www.umd.be/LMNA/>). Mutations on the genes encoding for other nuclear proteins as emerin, nesprins,... lead to similar alterations. The main mutations and the general characteristics of these pathologies are summarised in **Figure 4**. For this work, a special focus on *LMNA* gene mutations will be done.

The large spectrum of *LMNA*-laminopathies can be classified into four groups with some phenotypic overlapping 1) striated muscles diseases including *LMNA*-DCM-CD, EDMD, *LMNA*-related congenital muscular dystrophy (L-CMD) and limb-girdle muscular dystrophy type 1B (LGMD1B), 2) diseases affecting adipose tissue including familial partial lipodystrophy of Dunnigan type (FPLD), 3) peripheral neuropathy associated with demyelination of motor neurons such as axonal neuropathy Charcot-Marie-Tooth type 2B1 (CMT2B1), and 4) premature aging syndromes which include Hutchinson-Gilford progeria syndrome (HGPS) and atypical Werner syndrome (aWRN) (Bonne et al., 1999; Fatkin et al., 1999; Muchir et al., 2000; Shackleton et al., 2000; Sandre-Giovannoli et al., 2002; Chen et al., 2003; Eriksson et al.,

2003). Among all *LMNA*-laminopathies approximately 60% of the mutations involve striated muscle phenotype (Bertrand et al., 2011).

3.2.1 Striated muscle laminopathies

Emery-Dreifuss muscular dystrophy (EDMD) is a genetically heterogeneous disorder with X-linked, autosomal dominant and autosomal recessive forms. It is characterized by 1) early contractures of the Achilles tendons, elbows and post-cervical muscles, 2) slow progressive muscle wasting and weakness and 3) a cardiomyopathy usually presenting as cardiac conduction defects (Bonne et al., 1999) (Vigouroux and Bonne, 2013).

Limb-Girdle Muscular Dystrophy (LGMD) is a heterogeneous group of disorders that can be acquired in a dominant or recessive manner. It can present or not a cardiac alteration. In particular, LGMD associated with atrioventricular conduction disturbances (LGMD1B) is characterized by symmetrical weakness starting in the proximal lower limb muscles, and gradually proximal upper limb muscles also become affected. Mostly all patients present dysrhythmias and atrioventricular conduction defects. (van der Kooi et al., 1997; Muchir et al., 2000).

LMNA-associated congenital muscular dystrophy (L-CMD) is a myopathy due to *de novo* mutations. It appears in first year of life and can have different severity degrees. The main characteristics of this pathology are a selective axial weakness and wasting of the cervico-axial muscles, a limb muscle involvement and ventilator difficulties (needing a ventilator support) (Quijano-Roy Susana et al., 2008). Cardiac involvement was reported in some cases (i.e. severe ventricular arrhythmias associated with sudden cardiac death).

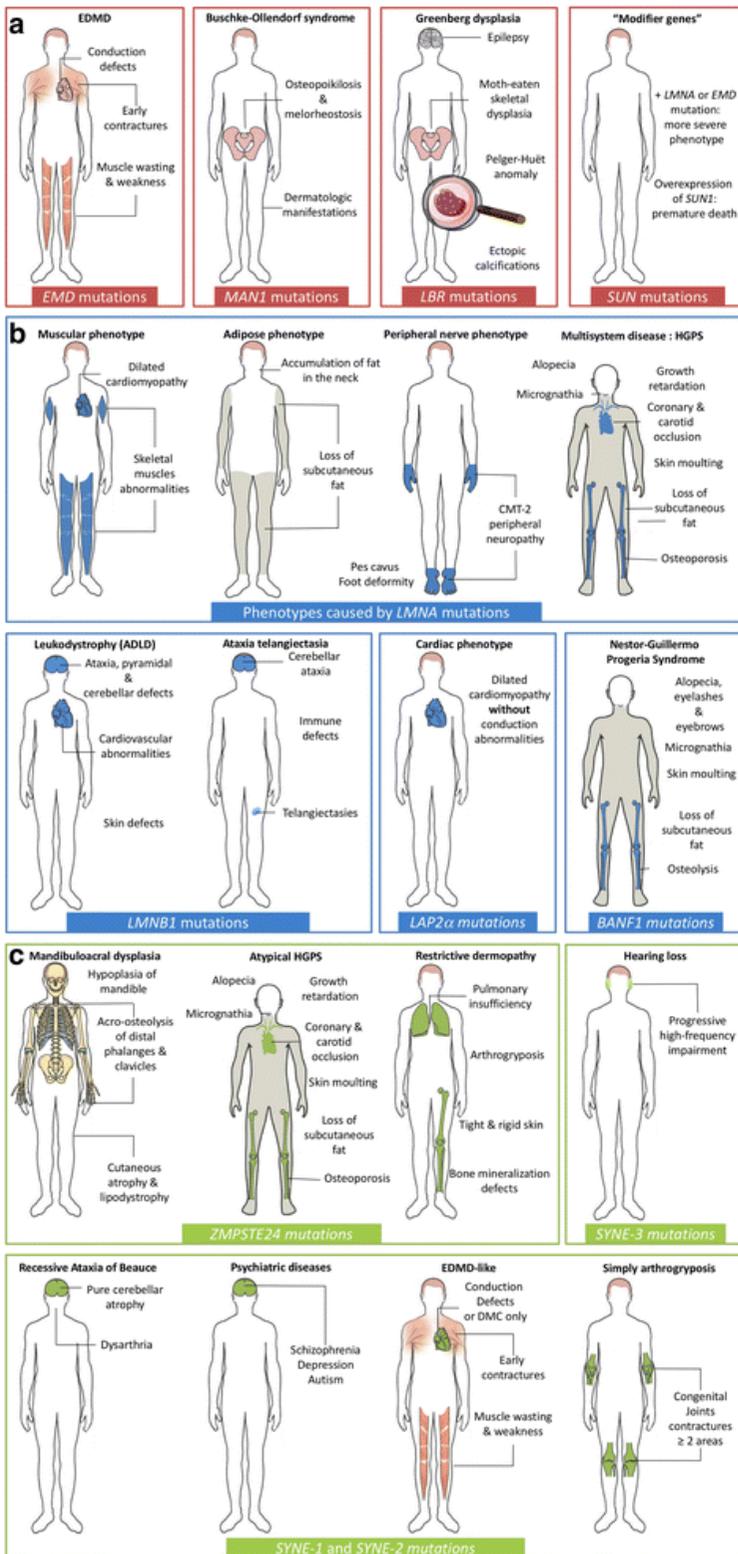


Figure 4: Summary of all known human diseases caused by mutations in genes coding for nuclear envelope components. The diversity of phenotypes induced by mutations in genes encoding nuclear envelope components, as well as the tissues affected by these, is illustrated and organized according to the localization of the mutated protein: **(a)** inner nuclear membrane, **(b)** nuclear lamina, and **(c)** outer nuclear membrane components. EDMD: Emery-Dreifuss Muscular Dystrophy, HGPS: Hutchinson-Gilford Progeria Syndrome, CMT: Charcot-Marie-Tooth Disease, ADLD: Autosomal Dominant Leukodystrophy.

Extracted from: Nuclear envelopathies: a complex LINC between nuclear envelope and pathology (Janin et al., 2017)

3.3 Lamins

Lamins are the major components of the nuclear lamina (NL). The NL is a protein meshwork underlying the inner aspect of the inner nuclear membrane known to serve as major structural component of the nucleus (**Figure 5**). Lamins provide a platform for the binding proteins and chromatin. They have varied roles in the cell, implicated in nuclear mechanical stability, chromatin organisation, DNA repair/replication, transcription, mediating cellular signalling and cytoskeletal interactions.

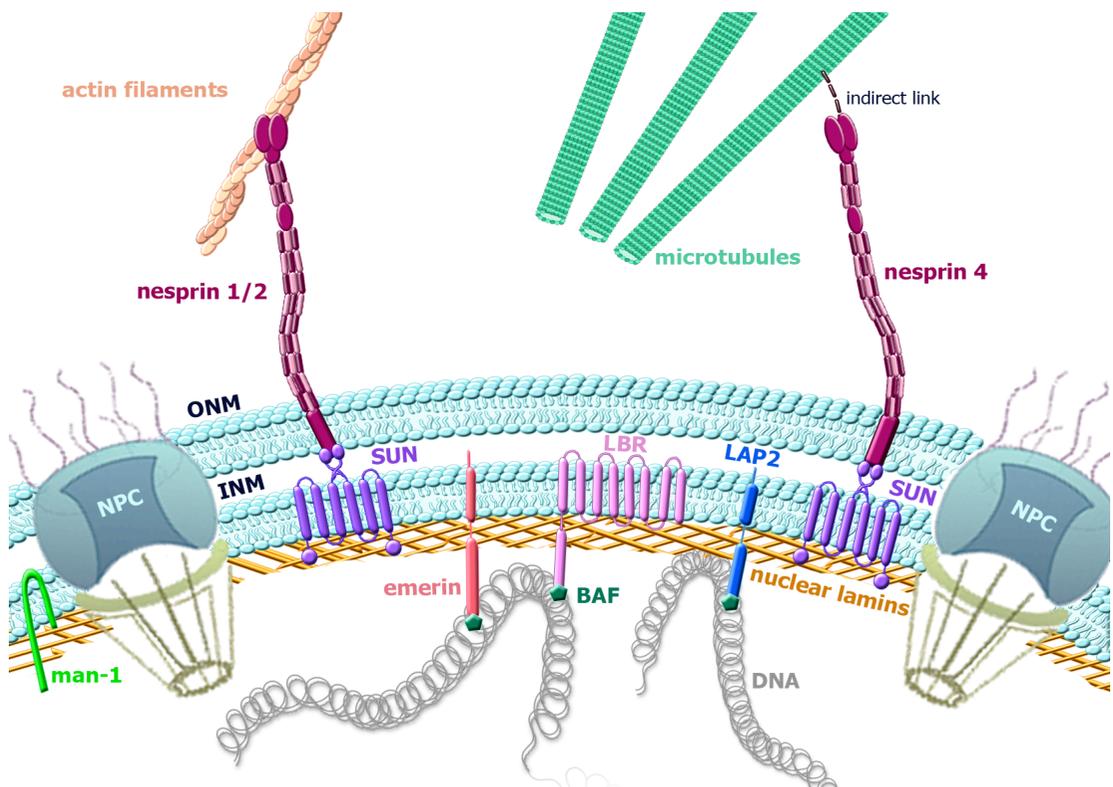


Figure 5: Schematic diagram of the nuclear envelope (NE) organization. NE is composed by an outer nuclear membrane (ONM) and an inner nuclear membrane (INM) joining at the nuclear pore complexes. Inserted into the NE, integral proteins MAN-1, SUNs, Emerin, LBR, LAP2, Nesprins display various interactions connecting the nucleoskeleton to the cytoskeleton.

3.3.1 Structure and genetics determinants of lamin proteins

Lamins are the only intermediated filaments located in the nucleus. As all intermediate filaments proteins, they possess an α -helical coiled-coil central rod domain, composed of heptad repeats of amino acids, bordered by a globular amino-terminal “head” domain and a carboxyl-terminal “tail” domain. This latter comprises an (Ig)-like domain and a nuclear localization sequence (Dhe-Paganon et al., 2002; Frangioni and Neel, 1993; Krimm et al., 2002; Parry et al., 1986).

After being processed, lamins form coiled-coil dimers (Aebi et al., 1986) that interact longitudinally through head-tail association to form a long polar polymer that can further assemble laterally forming 10nm intermediate filament proteins (Fisher et al., 1986; McKeon et al., 1986; Goldman et al., 1986). Recent studies are however challenging this view and described lamins as being 3.5nm filaments (Harapin et al., 2015; Turgay et al., 2017). This controversy would need further clarification.

Based on their sequences and structural properties nuclear lamins can be separated in two types: A-type and B-type lamins (Gerace, L, 1978), which form two separated filaments networks. A-type lamins are expressed in most differentiated somatic cells (Dechat et al., 2010) while B-type lamins are constitutively expressed. In mammals three genes encode for lamins: *LMNA*, *LMNB1* and *LMNB2*. *LMNA* is located to chromosome 1q21.2 (Wydner et al., 1996), it encodes for A-type lamins, which major isoforms are lamin A and C (Lin and Worman, 1993). These proteins are identical for their first 566 amino acids and vary in their carboxyl terminal tails due to alternative splicing. Lamins are synthesized as precursors that are post-translationally processed (Beck et al., 1990; Dhe-Paganon et al., 2002; Farnsworth et al., 1989; Maske et al., 2003; Nigg et al., 1992; Varela et al., 2005). Minor A-type lamins isoforms have been described such as lamin C2 and lamin A Δ 10 (Nakajima and Abe,

1995). The most abundant B-type lamins are lamin B1 and B2 respectively encoded by *LMNB1* and *LMNB2*, both being constitutively expressed. *LMNB2* encodes also lamin B3 by alternative splicing, which is restricted to the male germ line (Burke and Stewart, 2013; Gruenbaum et al., 2005).

3.3.2 Functional roles of lamin proteins

A. Nuclear envelope support

The nuclear envelope (NE) is a porous interface that separates the nucleus and the cytoplasm in eukaryotic cells (Aaronson and Blobel, 1975; Watson, 1955). Structurally this lipid bilayer consist in an outer nuclear membrane (ONM) and an inner nuclear membrane (INM) both enclosing the perinuclear space but joining at sites occupied by nuclear pore complexes (NPCs). NPCs are protein-based channels controlling the movement of molecules form the nucleus to the cytoplasm and vice versa. INM and ONM despite having continuous border they present several structural and functional differences.

The ONM is continuous with the endoplasmic reticulum membrane and shares some functions and structural features (Hetzer, 2010). Despite the continuity between this two membranes ONM contains several specific nuclear envelope transmembrane proteins (NETs) essential for the interaction with the cytoskeleton (Crisp et al., 2006; Starr and Fridolfsson, 2010; Gant Luxton and Starr, 2014). The INM interacts with the nucleoplasm through more than 100 NETs. Among all these proteins some are implicated in the assembling of nuclear factors that regulate transcription, cell division and DNA repair (Starr, 2011). In a general point of view INM and its associated NETs have two main roles, the interaction NE-chromatin and the interaction NE-NL (Czapiewski et al., 2016) (**Figure 5**). The interaction between the NE and the

NL started to be studied for their roles during mitosis (Moir et al., 2000; Panorchan et al., 2004) but have been demonstrated to be implicated in the maintaining of normal nuclear morphology and its composition (Gundersen and Worman, 2013). As structural components of the NL, A-type lamins confer viscosity and stiffness, while B-type lamins confer elasticity to the nucleus (Lammerding et al., 2006; Pajeroski et al., 2007). In addition to that, different mutations of lamins induce misshapen nuclei, nuclear pore clustering, mislocalisation of NETs and aberrant intranuclear foci, demonstrating the important role played by lamins in structural the structural nuclear support (Broers et al., 2004; Lammerding et al., 2004; Sullivan et al., 1999).

B. Lamin and chromatin

NL participates in chromatin organization, DNA repair and transcriptional regulation.

Chromatin organization

Lamins can organize and regulate chromatin position within the NE. They can interact in a direct or indirect manner (via lamin-binding proteins) with the chromatin (Camozzi et al., 2014; Gruenbaum and Foisner, 2015; Perovanovic et al., 2016). These dynamic interactions are essential to guide the spatial folding of the chromosomes (depending on the cell cycle state) and do not occur in all cell types identically (Guelen et al., 2008; Therizols et al., 2014). The direct interaction between lamins and chromatin occurs via specific genomic regions called lamina-associated domains (LADs). Contrarily indirect interactions can take place thanks to two families of INM integral proteins: LEM domain proteins family and Lamin B receptor proteins family. LEM domain proteins family is composed by: LAP2, emerin and MAN 1

domain, characterized by a 40 residues segment that interacts the Barrier-to-Autointegration Factor proteins (BAF). BAF proteins bind to double strand DNA and histones (Zheng et al., 2000). Through interactions with BAF proteins, LEM proteins contribute to the tethering of genomic regions to nuclear periphery, connecting interphase chromosomes to the nuclear lamina, thereby intervening in global nuclear organization. It is interesting to note that there is a 90% overlap between the LEM associated regions and LADs, suggesting that LEM proteins may contribute to LAD establishment or maintenance (Barton et al., 2014, 2015). The function of LEM proteins and their interactions with BAF and lamins are highly conserved through evolution, suggesting their essential role in scaffolding the NE.

Lamin B receptor proteins (LBR) are another family of INM integral proteins that contribute to the interactions of the nuclear lamina with the chromatin. These proteins bind to lamin B and chromatin during some cell phases (Olins et al., 2010). Their terminal domains give them their two main functions. The N-terminal domain tethers chromatin to the nuclear periphery, thus contributing to the shape of interphase nuclear architecture, while the transmembrane domains exhibit sterol reductase activity (Nikolakaki et al., 2017).

DNA repair

A-type lamins are implicated in: 1) the recruitment of the cell repair factor p53-binding protein (Liu et al., 2005; Varela et al., 2005), 2) in the protection against DNA damaging agents inducing single or double-stranded breaks (Liu et al., 2005; Masi et al., 2008; Richards et al., 2011), 3) in the control of ROS production and the sensitivity to oxidative stress (Richards et al., 2011) 4) in the regulation of Sirtuin 6 (SIRT6) activities (Ghosh et al., 2015) and 5) in the regulation of DNA repair foci positional stability (Mahen et al., 2013).

Mutations on A-type lamins have been associated with defective DNA repair. The progeria *Zmpste24*^{-/-} mouse model presents increased DNA damage, chromosome aberrations and higher sensitivity to DNA-damaging agents. In addition to that, the recruitment of some implicated-DNA-repair-proteins as Rad51 or 53p binding protein 1(53BP1) is impaired due to the mutation (Liu et al., 2005). Not only the recruitment of the 53BP1 is altered but all the stress signalling pathway marked by an upregulation of p53 target genes inducing a delayed checkpoint response and defective DNA repair (Varela et al., 2005; Masi et al., 2008). Contrarily the absence of lamin leads to 53BP1 degradation by the proteasome, showing its important role in the repair control (Gonzalez-Suarez et al., 2009). The absence of lamin induces slowed double strands breaks, with non-homologous end joining (Redwood et al., 2011).

The defects of DNA repair can also be highlighted in *Zmpste24*^{-/-} mouse model by an increased sensitivity to γ -irradiation (80% of the KO mice died compared to the 20% of the WT)(Liu et al., 2005). In fibroblasts carrying the A-type lamin-R527H mutation, the impairment of DNA repair was evidenced by an increased chromosome damage and the higher percentage of residual γ -H2AX foci (corresponding to unrepaired DNA-damage sites) (Masi et al., 2008). The fibroblast studies also showed an aberrant accumulation of Xeroderma Pigmentosum group A (XPA), a unique nucleotide excision repair protein (Liu et al., 2008) being partially responsible of the DNA repair deficiencies.

Transcription regulation

Nuclear lamins can modulate gene expression by direct interaction with chromatin or by sequestering transcriptional regulators at the nuclear periphery (Andrés and González, 2009; Reddy et al., 2008). During cells

differentiation, several genes located in LADs move away the NL and become activated or towards the NL being repressed (Therizols et al., 2014). Nevertheless different studies showed that the NL is not essential for this gene repression but rather increases the robustness of the repression (Yáñez-Cuna and van Steensel, 2017). In addition to that nuclear lamin can inhibit RNA polymerase II activity, (Spann et al., 2002).

Lamins also interact with some transcriptional regulators. A-type lamins have been shown to interact with Rb, Gcl, Mok2, cFos, and Srebp1, affecting gene expression either by a sequestration of these factors or by influencing the assembly of core transcriptional complexes (González et al., 2008; Kumaran et al., 2002; Malhas et al., 2009).

C. Mediating nucleo-cytoskeletal connections

Interactions with the cytoskeleton are essential for some of the nuclear lamin functions: nuclear positioning, nuclear shape and mechanosensing (Chang et al., 2015). These interactions involve proteins located in the INM and in the ONM among which KASH (Klarsicht, ANC-1, and Syne homology) and SUN (Sad1 and UNC-84) proteins, which form the linker of nucleoskeleton and cytoskeleton termed the LINC complex (Crisp et al., 2006; Gant Luxton and Starr, 2014; Starr and Fridolfsson, 2010).

Carboxyl terminus of A-type lamins interact with the LINC complex via SUN proteins in the nucleoplasm (Crisp et al., 2006; Haque et al., 2006). SUN-proteins are located in the INM and are encoded by SUN1 to SUN5, being SUN1 and SUN2 the main isoforms. SUN-proteins bind through the perinuclear space to KASH proteins interacting with cytoskeleton. The mammalian KASH proteins also called nesprins are located in the ONM, they are encoded by four different genes: *SYNE1*, *SYNE2*, *SYNE3* and *SYNE4*. Among all the isoforms

obtained by alternative splicing nesprin 1 α and nesprin 2 β interact with lamins (and emerin).

In mutant cells lacking lamin A, the mobility of the SUN proteins is increased, proving that lamin A plays a role in anchoring SUN proteins to INM (Östlund et al., 2009). In addition to that, during development B-type lamins play a role in SUN proteins anchoring (Chang et al., 2015). Other groups confirmed this anchoring function, reporting instability of TAN lines in cells lacking lamin A and nuclear migrations defects in cells with a weakened SUN-lamin interaction (Bone et al., 2014; Folker et al., 2011). Other factors also contribute to the SUN proteins anchoring because some cellular models lacking lamin present a normal SUN protein localization (Chang et al., 2015).

D. Mediating cellular signaling pathways

For almost 20 years now, it is known that proteins of the NL can modulate different signalling pathways due to their interactions with chromatin, cytoskeleton, LINC complex... These pathways have shown to be defective (altered/increased/decreased) in different laminopathies, explaining the important role of lamins. A-type lamins are involved in signalling pathways affecting cell growth, survival, migration and differentiation, including mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) pathways (Andrés and González, 2009; Bakay et al., 2006; Muchir et al., 2007a, 2007b).

More detailed signalling pathways implicated in *LMNA*-cardiomyopathy are described in 4.1.

The use of animal models has allowed a better understanding of these signalling pathways and has opened perspectives for new therapeutic approaches.

3.4 Animal models

To gain insight in the striated muscle laminopathies, different knock-out (KO), knock-in (KI) and transgenic mouse models have been developed.

While the use of KO mouse models are essential for the functional understanding of lamins, KI and transgenic mouse models allowed to understand the pathology and tissue specificity. All the actual striated muscle mouse models are presented in **table 4** and only the striated muscle *Lmna*-models are described below.

3.4.1 KO mouse model

The first animal model created was the *Lmna* KO mouse (*Lmna*^{-/-}). Even if there are no reported cases of human lacking lamin A/C (except an individual with haploinsufficiency of *LMNA* and foetus that died in gestation with an homozygous premature-stop-codon), this mouse model has been pivotal to determine the A-type lamins roles (Bonne et al., 1999; van Engelen et al., 2005; Muchir et al., 2003). This model has been one of the most used to understand the mechanics of lamins. *Lmna*^{-/-} mice present a dilated cardiomyopathy with conduction defects and skeletal myopathy similar to the one observed in patients with Emery-Dreifuss Muscular Dystrophy (Sullivan et al., 1999). *Lmna*^{-/-} mice at only 2 weeks of age present important growth defects and a reduced lifespan dying between 6 and 8 weeks of age. The heterozygous mice do not present any symptoms at early age but develop

atrio-ventricular conduction defects with atrial and ventricular arrhythmias (Wolf et al., 2008). However, even if this model was considered as “null” for many years, it was shown that the mice express a truncated form of *Lmna* (deletion of exons 8 to 11). The protein was detected at really low levels (mRNA and protein) but could act as a toxic molecule (gain-of-function) or as an hypoactive protein (loss-of-function) explaining the phenotype observed in this mice (Jahn et al., 2012). Three *KO* mice were latter developed: the *Lmna*^{GT-/-} mice (Kubben et al., 2011) the *Lmna*^{Δ/Δ} (Kim and Zheng, 2013) and the *Zp3-Lmna* (Solovei et al., 2013). The mice present severe growth retardation and cardiac developmental defects, myocytes hypertrophy and have a reduced life expectancy. They highlighted the implication of lamins in muscle development after birth (Kubben et al., 2011; Kim and Zheng, 2013; Solovei et al., 2013). While the use of *KO* mouse models are essential for the functional understanding of lamins, the *KI* and transgenic mouse model allowed to understand the instauration of the pathology and its tissue specificity.

3.4.2 KI and transgenic mouse model

The *KI* and transgenic mouse were developed from mutations found in patients with different Emery-Dreifuss Muscular Dystrophy. Two *KI* mouse models were developed almost simultaneously. Arimura *et al* developed the *Lmna*^{H222P/H222P}, created from a mutation found in patients with classical forms of EDMD. This model is characterized by a dilated cardiomyopathy with conduction defects and a progressive skeletal impairment leading to a premature death between 6 to 9 months for male and 7 to 13 for females. The cardiomyopathy is characterized by a left ventricular enlargement with a reduced fractional shortening and conduction defects with frequent sinoatrial blocks and ventricular extrasystoles (Arimura et al., 2005). The *Lmna*^{H222P/H222P} mouse model has been really useful to study the altered

signalling pathways (presented below). The skeletal phenotype had been less explored in those mice but similar signalling pathways are altered. Mounkes *et al* developed the *Lmna*^{N195K/N195K} mouse model, created from a missense mutation found in patients with dilated cardiomyopathy. The mice present a cardiac phenotype but no signs of skeletal muscle impairment. The cardiomyopathy is more severe than the one observed in *Lmna*^{H222P/H222P} mouse, and its accompanied by arrhythmias responsible of a premature death at 3 months of age (Mounkes et al., 2005). Wang *et al* developed the *Lmna*^{M371K} transgenic mouse model. This mutated lamin alters development that explains the increased prenatal loss and induces a severe cardiac phenotype that leads to a premature death at 2 to 7 weeks (Wang et al., 2006).

Bertrand *et al* developed a KI mouse model harbouring a *LMNA*-CMD; *Lmna*^{ΔK32/ΔK32}. This model exhibits altered striated muscle maturation and severe metabolic defects leading to a premature death at 2 weeks of age. Lamin proteins expression is reduced and delocalized, showing that the localization of lamins could be important for tissue maturation (Bertrand et al., 2012). The heterozygous *Lmna*^{ΔK32/+} mice do not present any initial phenotype with metabolic defects and die between 10 and 20 months of age. Nevertheless these mice presented a cardiac phenotype caused by the lamin A/C haploinsufficiency (Cattin et al., 2013).

The B-type mouse models develop different laminopathies such as Pelger-Huët anomaly, HEM-Greenberg skeletal dysplasia and ichthyosis reflecting the important role of lamin B in development. However these models no do not present a pathological muscular phenotype (Cohen et al., 2008; Shultz et al., 2003).

Model		Features	Reference
<i>Lmna</i> models			
KO	<i>Lmna</i> ^{-/-}	EDMD and DCM-CD	(Sullivan et al., 1999)
C.KO	<i>Lmna</i> ^{GT-/-}	Cardiomyocyte hypertrophy, skeletal muscle hypotrophy and metabolic defects.	(Kubben et al., 2011)
C.KO	<i>Lmna</i> ^{Δ/Δ}	Cardiomyocyte hypertrophy, skeletal muscle hypotrophy and metabolic defects.	(Kim and Zheng,
C.KO	<i>Zp3-Lmna</i>	Cardiomyocyte hypertrophy, skeletal muscle hypotrophy and metabolic defects.	(Solovei et al., 2013)
KI	<i>Lmna</i> ^{H222P/H222P}	AD-EDMD and DCM-CD	(Arimura et
KI	<i>Lmna</i> ^{pN195K/pN19}	DCM-1A -CD	(Mounkes
Tr	<i>Lmna</i> ^{p.M371K/p.M37}	EDMD and DCM	(Wang et al.,
KI	<i>Lmna</i> ^{ΔK32/ΔK32}	L-CMD, defective skeletal and cardiac muscles maturation and metabolic defects.	(Bertrand et al., 2011)
KI	<i>Lmna</i> ^{ΔK32/+}	DCM	(Cattin et
KI	<i>Lmna</i> ^{L530P/L530P}	Progeroid syndrome with growth retardation and heart underdevelopment and degeneration.	(Mounkes et al., 2003)
<i>Emd</i> models			
KO	<i>Emd</i> ^{-/-}	Muscle regeneration defects	(Melcon et
KO	<i>Emd</i> ^{-/-}	Motor coordination abnormalities, atrio-ventricular conduction defects	(Ozawa et al., 2006)
<i>KASH</i> models			
KO	^{Δ/Δ} <i>KASH</i>	DCM-CD	(Puckelwartz et al.,

Table 4: Mouse models for laminopathies.

4. Cellular and molecular alterations of cardiomyopathies: special focus on *LMNA*-cardiomyopathy.

4.1 Altered signalling pathways in *Lmna*^{H222P/H222P} and therapeutic approaches

The use of mouse models with cardiac defects due to *LMNA* mutations allows a better understanding of the pathology and improves the future treatments perspectives. The cardiac genome-wide RNA expression analysis performed by Muchir *et al*, on *Lmna*^{H222P/H222P} mouse revealed an alteration in several signalling pathways (Muchir et al, 2007).

4.1.1 MAP Kinase signalling

Mitogen activated protein kinase (MAPK) are a highly conserved family of serine/threonine protein kinases and are ubiquitously expressed signalling cascades, which transduce signal such as extracellular mitogens, growth factors and cytokines from the cell surface to the nucleus. MAPKs are involved in a variety of fundamental cellular processes such as proliferation, differentiation, motility, stress response and survival. Most common members of MAPKs family include the extracellular signal-regulated kinase 1 and 2 (Erk1/2 or p44/42), the c-Jun N-terminal kinases 1-3 (JNK1-3)/ stress activated protein kinases (SAPK1A, 1B, 1C), the p38 isoforms (p38 α , β , γ , and δ), and Erk5.

After the extracellular stimuli (mitogens, cytokines, growth factors, or environmental stressors) there is an activation of at least one of the MAPKK kinases (MAPKKKs) via receptor-dependent/independent mechanisms. Then MAPKKK phosphorylates and activates a downstream MAPK kinase (MAPKK), which in turn phosphorylates and activates MAPKs. Activation of MAPKs lead

to the phosphorylation and activation of specific MAPK-activated protein kinases (MAPKAPKs) that amplifies the signal and mediates the broad range of biological processes regulated by the different MAPKs. (Kim and Choi, 2010).

Alterations on the MAPKs pathway lead to severe cellular problems. Muchir *et al*, reported an hyperactivation of MAPK signalling pathway, opening a new field of therapeutic possibilities for laminopathies (Muchir et al., 2007b). Since this discovery, a deeper understanding of the alteration of this signalling pathway was done revealing an abnormal p38 α signalling (Muchir et al., 2012) an the direct effect of ERK 1/2 on CTGF/CNN2 expression (Chatzifrangkeskou et al., 2016) helping to understand the molecular signalling alterations of the *LMNA*-cardiomyopathy.

Nowadays different pharmacological inhibitors of the different actors of this pathway have been used as therapeutic approaches to alleviate the cardiac symptoms in *LMNA*-cardiomyopathy. Firstly a MEK 1/2 selective inhibitor, then a JNK inhibitor and the combination of both in *Lmna*^{H222P/H222P} mice, was shown to prevent left ventricular dilation and deterioration in cardiac contractility, to decrease the cardiac fibrosis and to prolong the lifespan (Muchir et al., 2012) (Muchir et al., 2009, 2010; Wu et al., 2011). The pharmacological inhibition of the p38 α has shown beneficial effects on the cardiac function allowing the first clinical trial in the disease, despite no major effects on cardiac fibrosis. These studies suggest that MAPKs pathways inhibition might constitute future therapeutic targets for the treatment of muscle-affected laminopathies.

4.1.2 TGF- β signalling

Transforming growth factor- β (TGF- β) superfamily signalling is one of the main regulators of cell growth, differentiation, and development. The signalling pathways are initiated by ligand-induced oligomerization of serine/threonine receptor kinases and a phosphorylation of the Smad2 and Smad3 (cytoplasmic signalling molecules), which could induce activation of two different pathways, the TGF- β /activin pathway, or Smad1/5/9 for the bone morphogenetic protein (BMP) pathway. The phosphorylation of the Smads leads to a partnering with the common signalling transducer Smad4, and translocation to the nucleus. The regulation of the different biological processes is done by the activated Smads via interactions with different transcription factors.

In the *Lmna*^{H222P/H222P} mouse, the presence of fibrosis and an increase of TGF- β signalling were reported (Arimura et al., 2005). This signalling pathway has been also reported to be altered in lipodystrophies caused by LMNA mutations (Le Dour et al., 2017a). Pharmacological inhibition of the TGF- β type I receptor kinase in the *Lmna*^{H222P/H222P} mouse model reduced myocardial fibrosis, reduced left ventricular diameters and increased left ventricular fractional shortening (Chatzifrangkeskou et al., 2016).

4.1.3 Wnt- β catenin signalling

Wnt signalling encompasses multiple and complex signalling cascades and is involved in many developmental processes such as tissue patterning, cell fate specification, and control of cell division. (Logan and Nusse, 2004). When Wnt is absent the β -Catenin suffers different posttranslational modifications that induce its degradation in the proteasome. When Wnt is present, the interactions with different proteins allow the accumulation of β -Catenin in the

cytoplasm and its translocation to the nucleus. B-catenin nuclear import induces the activation of Wnt target genes (MacDonald et al., 2009).

Wnt signaling has been reported to be altered in mouse models presenting skeletal and cardiac muscle alterations as progeroid syndromes but also in patients with *LMNA*-cardiomyopathy (Hernandez et al., 2010; Le Dour et al., 2017b).

4.1.4 AKT/mTOR signalling

mTOR (target of rapamycin (Laplane and Sabatini, 2012)) is a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and interacts with several proteins. The AKT/mTOR pathway responds to diverse environmental stimuli (growth factors, aminoacids, oxygen, stress...) and is acutely sensitive to rapamycin. AKT/mTOR pathway controls many processes that generate or use large amounts of energy and nutrients. Its main roles are the regulation of cell growth and proliferation. The AKT/mTOR signalling pathway has also been identified as implicated in the development of *LMNA* cardiomyopathy. It was reported to be hyperactivated in two different mouse models (in *Lmna*^{H222P/H222P} and *Lmna*^{-/-} mice) with alteration of the autophagic response. The inhibition of the AKT/mTOR signalling via rapamycin enhances the heart function of the *LMNA* mutants and blocks molecular cardiac remodelling (Choi et al., 2012; Ramos et al., 2012). However, ATK being part of the equation, other treatments inhibiting both ERK1/2 and AKT pathways were performed in these studies but did not improve cardiac function (Choi et al. 2012). The exact mechanism by which defects in A-type lamins lead to dysregulation of ERK1/2 and AKT pathways, remain unknown.

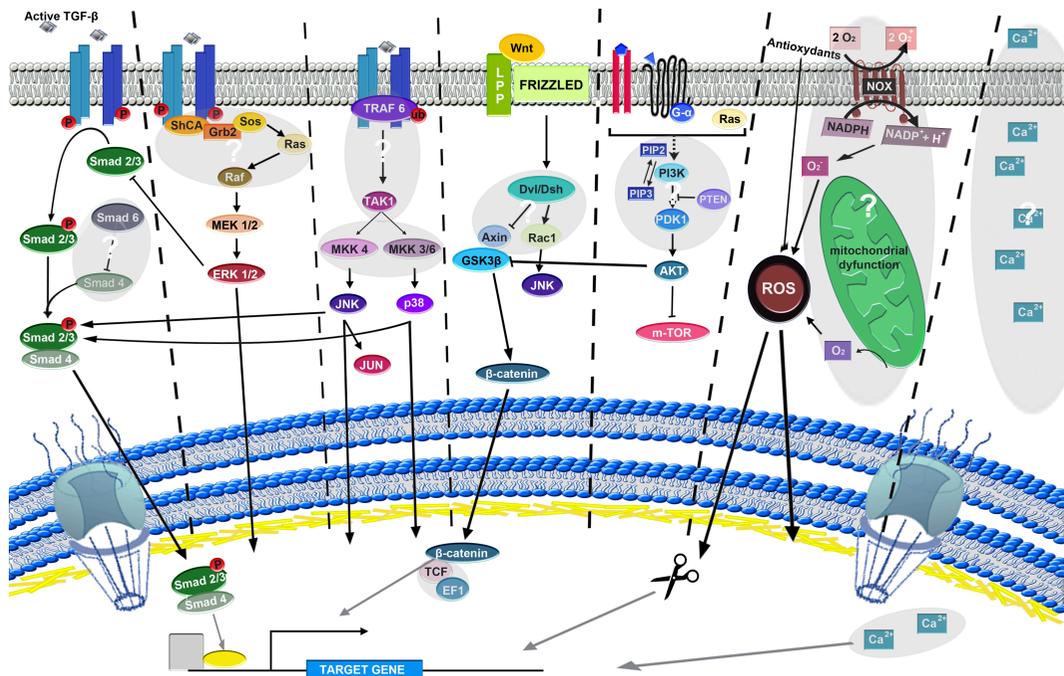


Figure 6: Altered signalling pathways in skeletal muscle laminopathies. Recapitulation of currently described signalling pathways implicated in the skeletal muscle laminopathies and (under grey shadow) recapitulation of other possible proteins implicated in this signalling pathways that has already been described in other pathologies.

4.2 Other therapeutical approaches

4.2.1 Oxidative stress

Reactive oxygen species (ROS) are produced as a result of normal cellular metabolism mediating various cellular responses including cell proliferation, differentiation, gene expression and migration. However, at high concentrations, ROS produce adverse modifications to proteins, lipids and DNA. In normal conditions, the cell can control this over-production with an antioxidant protection system composed by enzymes as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) and non-

enzymatic systems as glutathione (GSH) and vitamins. Oxidative stress reflects an imbalance between systemic manifestation of ROS and the ability of a biological system to readily detoxify the reactive intermediates or to repair the resulting damage. The 3 majors ROS are superoxide anion (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2) (Birben et al., 2012). They can be produced by different enzymes such as the NADPH oxidase family expressed in most mammalian cells. ROS generated by NADPH oxidases have crucial roles in various physiological processes, including innate immunity, modulation of redox-dependent signalling cascades, and as cofactors in the production of hormones (Drummond et al., 2011). The accumulation of ROS induces different modifications of DNA as degradation of single bases, single or double-stranded DNA breaks, mutations and can alter gene expression through epigenetic (via DNA-methylation) or genetic mechanisms (via the activation of redox sensitive factors as Rb, p53, FoxO and NFκB) (Allen and Tresini, 2000).

Oxidative stress is highly relevant in many pathological conditions including cancer, neurological disorders, cardiomyopathies, laminopathies... (Birben et al., 2012; Sieprath et al., 2012, 2015). Excessive accumulation of ROS has been reported in pathologies induced by *LMNA* mutations as lipodystrophies (Caron et al., 2007a; Verstraeten et al., 2009), amyotrophic quadricital syndrome (Charniot et al., 2007), HGPS (Viteri et al., 2010) and AD-EDMD (Pekovic et al., 2011). ROS accumulation can be explained by an altered DNA repair due to mutated lamins or an altered signalling mechanism having a secondary effect on the over-production of ROS. Reducing the excessive accumulation of ROS has been successfully used as treatment in progeria (Richards et al., 2011). Same type of treatment has also been used in inherited cardiomyopathies (Dludla et al., 2017; Wilder et al., 2015).

Targets of redox signaling in the heart		
Target	Mecanism	Reference
Ca ²⁺ /Calmodulin-Dependent Kinase II	Intersubunit autophosphorylation at Thr-287 within the autoinhibitory domain, preventing its reassociation with the catalytic domain and sustaining kinase activity even when Ca ²⁺ levels decline	(Hudmon and Schulman, 2002)
cAMP-Dependent Protein Kinase A (PKA)	Regulated through the formation of 2 interprotein disulfides in the regulatory R1 α subunits	(Brennan et al., 2006)
cGMP-Dependent Protein Kinase G (PKG)	Allosteric binding of cGMP activates the kinase. PKG1 α can be oxidized to form an intermolecular disulfide between its 2 Cys42 residues within the dimerization domain, activating the enzyme independently of the NO-cGMP pathway	(Burgoyne et al., 2007)
RyR2	Normally phosphoregulated by PKA and CaMKII. It can be subject to cysteine thiol oxidation	(Xu et al., 1998)
SERCA2a	Normally regulated by phospholamban can be phosphorylated by PKA or CaMKII. Complex regulation: low oxidative stress induces the activation of SERCA, however high concentration of oxidative stress inhibits SERCA. Other post-translational modifications can also be regulated by ROS like S-gluthathionylation or sulfination regulating its activity.	(Zima and Blatter, 2006)
NCX	Normally phosphoregulated by PKA and PKC but is also redox modulated via direct oxidation of sensitive cysteines.	(White et al., 2009)
Histones deacetylases	Histone acetylation promotes and deacetylation inhibits gene expression, these processes being regulated by histone acetyltransferases and histone deacetylases (HDACs), respectively.	(Ago et al., 2008)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Catalytic thiol is subject to reversible and irreversible forms of inhibitory oxidation. The protein can be S-nitrosylated to trigger nuclear translocation and apoptosis	(Eaton et al., 2002)

Table 5: Recapitulation of the main cardiac targets of redox signalling.

4.2.2 Calcium imbalance

Calcium homeostasis is essential for cardiac function and main calcium regulators are summarised in **table 6** Different alterations on the expression of calcium channels and their regulators have been reported to be involved in the development of dilated cardiomyopathies. Mutations on phospholamban, the main ventricular regulator of the SERCA2a pump, are one of the main

causes of familial dilated cardiomyopathy (Mestroni et al., 2014). In what *LMNA*-cardiomyopathy is concerned, no calcium alterations have been reported to the date. Nevertheless, a genome-wide RNA Array study reported an important alteration of the genes encoding for sarcolipin and L-type channels, suggesting a possible dysregulation of the calcium balance (Muchir et al., 2007b). In addition, Arimura *et al* used Ca^{2+} sensitizers to treat the *Lmna*^{H222P/H222P} mouse model, reporting an improved systolic dysfunction, prolonged life expectancy and decreased cardiac interstitial fibrosis (Arimura et al., 2010). Calcium sensitizers are molecules that modulate the contractile force without inducing any change in the calcium transient.

Although the underlying mechanism remains unknown, the beneficial effects of the treatment suggest that the Ca^{2+} sensitizers could be an option for preventing the progression of *LMNA* cardiomyopathy and the putative roles of calcium in the development of the pathology need further investigation.

	Function	Gene	Regulated by	Location	Size
L-type channel	Allows the entrance of Ca^{2+} from the extracellular medium to the intracellular medium (initiates E-C coupling inducing the CICR)	<i>CACNA1</i> , <i>CACNB2</i> , <i>CACNA2D</i>	Membrane depolarisation and Calmodulin (CaM)	Sarcolemma (specially in T-tubules)	430 KDa
NCX1	Allows the extrusion of the cytosolic Ca^{2+} helping in the relaxation (ending the E-C coupling)	<i>SLC8A1</i>	Na^+ , Ca^{2+} , protons, PIP2 , PKA, exogenous agents	Sarcolemma	110 KDa
RyR2	Responsible of intracellular Ca^{2+} release	<i>RYR2</i>	L-type channels, Ca^{2+} , Mg^{2+} ,PKA, FKBP12 and 12.6, CaM, CaMKII, CSQ, triadin and junctin	SR membrane	>2 MDa (each subunit is >550 kDa)
SERCA2a	Responsible of translocation of Ca^{2+} from the cytosol into the SR lumen	<i>ATP2A2</i>	phospholamban and sarcolipin	SR membrane	115 KDa

Table 6: Main calcium cardiac channels.

4.3 Sarco(endo)plasmic reticulum calcium ATPase and its inhibitors

The sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) is an essential Ca^{2+} -handling protein implicated in the relaxation of the muscle via the recapture of the cytosolic Ca^{2+} (Møller et al., 2010). In cardiomyocytes, SERCA₂ is responsible for the removal of 70-90% of Ca^{2+} from the cytosol, depending of the species (Milani-Nejad and Janssen, 2014). This process, coupled to other Ca^{2+} transport mechanisms, allows for cardiac muscle relaxation (diastole) (Bers, 2002). The mutations of the genes encoding the different SERCA isoforms lead to a variety of pathologies (Hovnanian, 2007; Lipskaia et al., 2014) highlighting their important role in normal cardiac function. In the heart, the major isoform is the SERCA2a and its dysregulation has been described as a major contributing factor for the progression of heart failure. SERCA2a constitutes a therapeutic target of interest and clinical trials aiming at promoting its activity have been performed for more than a decade (Kho et al., 2011; Park and Oh, 2013; Shareef et al., 2014).

SERCA2a is a 993 amino acid protein composed by a transmembrane domain and a large cytoplasmic head. The transmembrane domain contains the high affinity sites where the Ca^{2+} ions bind for the transport towards the SR. This transmembrane domain is composed of several α -helices connected with three cytosolic domains. These three domains are: the ATP binding N-domain, the P-domain that gets phosphorylated by the γ -phosphate of ATP, and the A-domain that coordinates the de-phosphorylation (Toyoshima et al., 2000). SERCA2a transports two Ca^{2+} ions across SR membrane by interconverting between high (E1) and low (E2) ion-affinity states. During E1 state two ions bind to the high affinity sites (I and II) that open towards the cytosol. Then, movements of the cytosolic domains driven by ATP hydrolysis, phosphorylation and dephosphorylation change the affinity of the Ca^{2+} binding

sites for directional transfer of Ca²⁺ into the SR lumen and lead to the formation of the Ca²⁺-free state (E2). The kinetics cycle of SERCA are represented in Figure 6.B, (extracted from Shaikh *et al.* 2016). SERCA2a can be regulated by post-translational modification (summarised in **table 7**), hormones like thyroid hormones (Trivieri *et al.*, 2006) or adiponectin (Altamirano *et al.*, 2015), miRNAsm like miR-22 (Gurha *et al.*, 2012) or miR-25 (Wahlquist *et al.*, 2014) and by different proteins presented below.

Post-translational modification of SERCA		
Modification	Effect	Ref
Glutathiolation	It leads to an enhanced SERCA activity, which augments the Ca ²⁺ influx.	(Adachi <i>et al.</i> , 2004; Tong <i>et al.</i> , 2008; Lancel <i>et al.</i> , 2009)
	Oxidative stress can inhibit permanently this post-translational modification.	(Jardim-Messeder <i>et al.</i> , 2012)
Nitration	Its role is not well elucidated. The nitration of the SERCA pump has been observed in failing hearts, hypothesizing a possible pathological role of it.	(Ide <i>et al.</i> , 1999; Viner <i>et al.</i> , 1999; Nakamura <i>et al.</i> , 2002; Lokuta <i>et al.</i> , 2005; Tang <i>et al.</i> , 2010)
SUMOylation	It promotes contractile properties and reduces pressure overload in failing hearts. SUMOylation has been hypothesized as a cardioprotective post-translation modification.	(Kho <i>et al.</i> , 2011, 2015)
Acetylation	It leads to an alter Ca ²⁺ influx. The acetylation of the SERCA pump has been prominently observed in failing hearts, hypothesizing a possible pathological role of it.	(Foster <i>et al.</i> , 2013)
	This post-translation modification is reversed by (SIRT1) deacetylase	(Kho <i>et al.</i> , 2011)
Glycosylation	It reduces the activity of SERCA2a and Ca ²⁺ efflux. This post-translational modification susceptible to occur at elevated glucose levels possibly being implicated in the relationship between diabetes and heart failure.	(Clark <i>et al.</i> , 2003; Bidasee <i>et al.</i> , 2004; Ballal <i>et al.</i> , 2010)
	O-GlcNAcylation is the most studied SERCA glycosylation due to its link to different pathologies such as aging, ischemia and heart failure. This regulation can be done directly or indirectly, altering PLN phosphorylation.	(Hu <i>et al.</i> , 2005; Zachara and Hart, 2006; Young <i>et al.</i> , 2007; Fülöp <i>et al.</i> , 2008; Champattanachai <i>et al.</i> , 2008; Watson <i>et al.</i> , 2010; Bennett <i>et al.</i> , 2013)

Table 7: SERCA post-translation modifications.

Sarcoplipin (SLN) and phospholamban (PLN) are the two major inhibitors of SERCA2a. They present similar structures and the same function. Nevertheless, their expression pattern and their mode of inhibition are not the same. In the heart, in normal conditions, SLN is expressed predominantly in the atria contrarily to PLN mostly expressed in the ventricles. In pathological conditions this expression pattern can be altered.

4.3.1 Phospholamban

A. Structure

Phospholamban (PLN) is a 52-aminoacid protein organised in three domains: two cytosolic domains Ia and Ib and one transmembrane domain (**Figure 7A**). Two phosphorylation sites are located in the Ia domain (residues 1-20) in control of PLN activity: Serine16 (Ser16), phosphorylated by PKA and Threonine17 (Thr17) phosphorylated by Ca²⁺/CaM kinase (Simmerman et al., 1986). The cytosolic domain Ib is rich in amidated amino acids and is responsible of the interaction with SERCA (Asahi et al., 2001). The domain II (residues 31-52) is composed of nonpolar and hydrophobic amino acids, forming a transmembrane helix (Jones et al., 1985).

Five PLN can assemble by parallel packing into pentamers, either monomers or pentamers can interact via the transmembrane domain with the SERCA pump (Oxenoid and Chou, 2005).

B. SERCA inhibition

PLN inhibits SERCA activity by decreasing Ca²⁺ affinity at its two Ca²⁺ binding sites (I and II) located in the transmembrane domain. Phosphorylation of PLN at Ser16 and Thr17 partially reverses PLN induced alterations in Ca²⁺ affinity, increasing contractility (Akin et al., 2013). PLN can exclusively link to SERCA and decrease the Ca²⁺ affinity through direct effects on the Ca²⁺ binding

sites (Jones et al., 2002).

C. Pathological implication

Alterations on PLN protein can lead to dilated and hypertrophic cardiomyopathies. The dilated cardiomyopathies due to PLN mutations are characterized by an early onset of the pathology with increased chamber dimensions, decreased contractile function at age 20 to 30 years, and progression to heart failure within 5 to 10 years after symptom onset. The congestive heart failure can be severe needing a cardiac transplantation. The hypertrophic cardiomyopathy is rarer than the dilated cardiomyopathy and its exclusively due to mutations in the promotor of the PLN (Haghighi et al., 2003, 2006, 2008; Medin et al., 2007; Minamisawa et al., 2003; Schmitt et al., 2003; Chiu et al., 2007). Different therapeutic strategies are being developed to regulate the activity of the protein, e.g. protein interacting RNAs and XNAs (oligonucleotide analog) (Soller et al., 2016).

4.3.2 Sarcolipin:

A. Structure

Sarcolipin is a type I integral protein composed by 31 amino-acids. These 31 residues form three different domains: a hydrophobic N-terminal domain located in the cytoplasm (residues from 1 to 7), a single transmembrane domain (residues from 8 to 26) and a luminal C-terminal domain (residues from 27 to 31)(**Figure 7A**) (Wawrzynow et al., 1992; Odermatt et al., 1997). The N-terminal domain contains two possible phosphorylation sites: serine4 (Ser4)(in mice) and threonine5 (Thr5)(in all mammals), (Bhupathy et al., 2009; Shanmugam et al., 2015); the transmembrane domain contains residues involved in inhibitory function (Odermatt et al., 1998) and the C-terminal domain is essential for its retention in the SR and assures SERCA inhibition

(Gramolini et al., 2004; Hughes et al., 2007; Gorski et al., 2013) RSYQY residues are conserved in all species located in the C-terminal domain assuring the regulating role of sarcolipin (Gorski et al., 2013). The transmembrane domain presents 84% homology with the PLN sequence suggesting similar linking to the SERCA pump.

As PLN, SLN can be found as a monomer or as a pentamer. SLN monomers link to SERCA but can also self-associate into dimers and higher order oligomer in the absence of SERCA (Autry et al., 2011; Cao et al., 2016).

B. SERCA inhibition

Different studies showed that SLN inhibits SERCA by lowering Ca^{2+} affinity and decreasing its maximal activity (V_{max}). The inhibition is done *via* an interaction with SERCA in the E1 and E2 states, indicating that contrarily to PLN its inhibition is not reversed at high calcium concentrations (**Figure 7B**) (Tupling et al., 2002; Babu et al., 2006; Hughes et al., 2007; Sahoo et al., 2013; Shaikh et al., 2016; Sahoo et al., 2015). The regulatory effect of SLN can be reversed by β -adrenergic response (Babu et al., 2006; Bhupathy et al., 2007, 2009).

PLN and SLN can occupy the same interaction site with SERCA (Asahi et al., 2003b), nevertheless there is no competition between the two proteins. SLN can bind directly to PLN preventing its polymerization inducing a super inhibitory effect of PLN to SERCA (Asahi et al., 2002; Vangheluwe et al., 2005).

C. Sarcolipin a thermogenic regulator

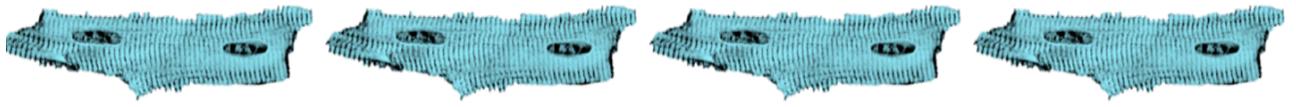
SLN plays an important role in muscle thermogenesis and metabolism. SLN inhibition induces ATP hydrolysis and decreases Ca^{2+} transport into the SR is due to 'slippage' of Ca^{2+} back to the cytosol. In the presence of SLN, more ATP needs to be hydrolysed by SERCA to transport the released Ca^{2+} than in the absence of it. Although *in vitro* the presence of SLN leads to an increased

heat level consistent with the ATP hydrolysis its specific role is still unclear (Smith et al., 2002; Mall et al., 2006; Sahoo et al., 2013). *In vivo* Bal et al reported that SLN is necessary for muscle-based thermogenesis due to the incapacity of *Slh*^{-/-} mice to maintain their body temperature when challenged to acute cold, suggesting a role of SLN in the non-shivering thermogenesis (Bal et al., 2012).

SLN is also implicated in the regulation of whole-body metabolism and weight gain in mice (Bal et al., 2012; Sahoo et al., 2013; Maurya et al., 2015). The regulation of muscle metabolism and energy expenditure is done via its cytosolic domain providing resistance against diet-induced obesity and extreme cold (Autry et al., 2016). Nevertheless the mechanism is still unclear and further work is necessary to understand the detailed mechanism by which SLN regulates energy expenditure in muscle.

D. Pathological implication

Up-to-date no *SLN* mutation has been described as responsible of any human pathology. However its expression is altered in different pathologies as dysferlinopathy, atrial fibrillation, obesity and EDMD (Shanmugam et al., 2011; Vittorini et al., 2007; Voit et al., 2017). The studies in animal models showed that mutations or dysregulation of SLN can contribute to contractility defects. The overexpression of SLN leads to a decreased cardiomyocyte contractility and decreased calcium transient (Asahi et al., 2004; Babu et al., 2005).



Results

Chapter 2: Results

This chapter is composed by two peer-reviewed articles.

N-acetyl cysteine alleviates oxidative stress and protects mice from dilated cardiomyopathy caused by mutations in nuclear A-type lamins gene .

Blanca Morales Rodríguez#, Lara Khouzami#, Valérie Decostre, Shaida Varnous, Vanja Pekovic-Vaughan, Christopher J. Hutchison, Françoise Pecker, Gisèle Bonne, Antoine Muchir

This first article focuses on the role played by oxidative stress in development of *LMNA* cardiomyopathy and the possible therapeutic strategy based in the reestablishment of normal glutathione levels by a N-acetyl cysteine treatment.

Activation of sarcolipin expression links mutations in A-type lamins genes to cardiomyopathy

Blanca Morales Rodríguez, Aurore Besse, Nathalie Mougnot, Florence Lefebvre, Jean-Pierre Benitah, Ana-María Gómez, Véronique Briand, Philippe Beauverger, Gisèle Bonne, Antoine Muchir

This second article focuses on the role played by sarcolipin, in development of *LMNA* cardiomyopathy and the consequences of its over-expression in intracellular calcium homeostasis.

N-acetyl cysteine alleviates oxidative stress and protects mice from dilated cardiomyopathy caused by mutations in nuclear A-type lamins gene

(Published in Human Molecular Genetics, June 2018)

Introduction

LMNA cardiomyopathy is characterized by left ventricular dysfunction and dilatation, conduction defects, arrhythmias, often leading to heart failure-related disability. There is currently no treatment for *LMNA* cardiomyopathy and heart transplantation is often the last therapeutic option. Oxidative stress has been described in different cardiac pathologies as an important contributor of the disease progression. However, whether oxidative stress is altered in dilated cardiomyopathy caused by mutations in nuclear A-type lamins gene remains unknown.

Objectives of the study

The aim of this study was to determine the possible implication of oxidative stress in the progression of *LMNA* cardiomyopathy.

Materials and methods

To determine the implication of oxidative stress in the *LMNA* cardiomyopathy progression, we have used the *Lmna*^{H222P/H222P} mouse model. We have analyzed several oxidative stress markers (protein carbonylation, NADPH oxidases, antioxidant proteins) in hearts from *Lmna*^{H222P/H222P} mice. To test a new therapeutic approach, we have studied the effect of N-acetyl cysteine (glutathione precursor) on left-ventricular function and fibrosis of *Lmna*^{H222P/H222P} mice.

Results

We showed that *LMNA* cardiomyopathy was associated with altered oxidative stress characterized by an imbalance between the ROS producers and the antioxidant systems. Particularly, we observed a decreased expression of the key cellular antioxidant: the glutathione. We next treated *Lmna*^{H222P/H222P} mice with N-acetyl cysteine, a glutathione precursor, administered orally for one month. Decreasing cardiac oxidative stress, this treatment led to an improvement of left ventricular structure and function,

Impact

In conclusion, our experiments demonstrate a novel contributory mechanism for *LMNA* cardiomyopathy triggered by oxidative stress and impaired antioxidant protection. Because N-acetyl cysteine pharmacodynamics and absence of toxicity are well established, our work supports further studies on humans to begin to evaluate the therapeutic benefits of lowering oxidative stress and assessing the benefit of such therapy in cardiomyopathy. Overall, our work points toward clinically relevant N-acetyl cysteine as a possible future treatment option for patients with *LMNA* cardiomyopathy in addition to standard therapy.

N-acetyl cysteine alleviates oxidative stress and protects mice from dilated cardiomyopathy caused by mutations in nuclear A-type lamins gene

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ABSTRACT

Cardiomyopathy caused by lamin A/C gene (*LMNA*) mutations (hereafter referred as *LMNA* cardiomyopathy) is an anatomic and pathologic condition associated with muscular and electrical dysfunction of the heart, often leading to heart failure-related disability. There is currently no specific therapy available for patients that target the molecular pathophysiology of *LMNA* cardiomyopathy. We showed here an increase in oxidative stress levels in the hearts of mice carrying *LMNA* mutation, associated with a decrease of the key cellular antioxidant glutathione. Oral administration of N-acetyl cysteine (NAC), a glutathione precursor, led to a marked improvement of glutathione content, a decrease in oxidative stress markers including protein carbonyls and an improvement of left ventricular structure and function in a model of *LMNA* cardiomyopathy. Collectively, our novel results provide therapeutic insights into *LMNA* cardiomyopathy.

Keywords

LMNA, A-type lamins, cardiomyopathy, oxidative stress, glutathione, N-acetyl cysteine

INTRODUCTION

Dominant mutations in the gene encoding nuclear A-type lamins (*LMNA*) cause dilated cardiomyopathy with conduction system disease (*LMNA* cardiomyopathy) (1,2). *LMNA* cardiomyopathy is characterized by conduction defects, arrhythmias, left ventricular dysfunction and dilation, often leading to heart failure-related disability (3-5). *LMNA* cardiomyopathy can be associated with muscular dystrophy (Emery-Dreifuss muscular dystrophy (EDMD) and the limb-girdle muscular dystrophy type 1B (LGMD1B)) (5). The management of the cardiac disease consists in controlling arrhythmia and conduction defects by implantation of a defibrillator as patients are at high risk of sudden cardiac death (3). This limits the progression of heart failure, but eventually patients require heart transplantation. There are currently no effective approaches to treat *LMNA* cardiomyopathy in the clinic. Thus, it is of great importance to clarify its molecular mechanisms and to search for potential compound to provide protection against *LMNA* cardiomyopathy.

In order to study the pathophysiology of *LMNA* cardiomyopathy and to test possible therapeutics, we have developed a mouse model carrying a missense *LMNA* mutation, leading to the substitution of the histidine 222 by a proline (H222P), identified in a family with *LMNA* cardiomyopathy (6). Homozygous mutant (*Lmna*^{H222P/H222P}) mice develop cardiac left ventricular dilation and systolic dysfunction and have a reduced life expectancy. We previously identified and studied multiple mechanisms that contribute, in part, to the pathophysiology of *LMNA* cardiomyopathy. These include abnormal regulation of the mitogen-activated protein kinase (MAPK) signaling cascade (7,8), altered Wnt/ β -catenin signaling (9), fibrosis (10), AKT/mTOR signaling (11) and calcium handling (12). All these signaling mechanisms have been targeted with small-molecule inhibitors in

Lmna^{H222P/H222P} mice and all of these interventions have had some beneficial impact. However, targeting signaling of each of these pathways alone is not curative (13).

Searching for alternative ways to slow down the cardiac disease progression, we here examined the involvement of oxidative stress in the progression of the cardiac disease in *Lmna*^{H222P/H222P} mice. Recent data point to a relationship between lamin mutations and altered reactive oxygen species (ROS) metabolism (14). ROS are small, short lived signaling molecules that mediate various cellular responses (15). Excessive accumulation of ROS can lead to DNA damage and the build-up of oxidized proteins and lipids. The major intracellular sources of ROS are the mitochondria and the NADPH oxidases (NOX). To counter the potential damaging effects of ROS, cells have evolved several antioxidant systems, including ROS alleviating enzymes like catalase and glutathione peroxidase (GPX), as well as non-enzymatic systems comprising glutathione (GSH).

Herein, we show that the cardiomyopathy in *Lmna*^{H222P/H222P} mice is associated with altered oxidative stress levels and glutathione deficiency. Accordingly, glutathione replenishment with N-acetyl cysteine (NAC) treatment reduces cardiac oxidative stress injury, and ameliorates contractile dysfunction in *Lmna*^{H222P/H222P} mice.

RESULTS

***Lmna*^{H222P/H222P} mice with cardiac dilatation display increased oxidative stress markers**

To explore the role of oxidative stress pathway in the development of dilated cardiomyopathy, we studied a mouse model that present a *Lmna* mutation substituting the histidine in position 222 into a proline (6). The male mice develop a progressive contractile dysfunction, cardiac remodeling and die by 32-34 weeks of age. Similar features were

observed in female *Lmna*^{H222P/H222P} mice but with a later-onset (6). *Lmna* p.H222P corresponds to a human disease-causing mutation associated with dilated cardiomyopathy (16). Given that a consequence of altered ROS metabolism would lead to an aberrant cardiac oxidative stress (Figure 1A), we first assessed the steady-state cardiac protein carbonylation level in *Lmna*^{H222P/H222P} mice. We showed that the protein carbonylation content was increased in hearts from *Lmna*^{H222P/H222P} mice compared to wild type mice, when the left ventricular function was altered at 6 months of age (symptomatic) (Figure 1B).

Since oxidative stress can be triggered by activation of NADPH oxidases (NOXs), we examined NOX2 expression in the hearts of 6-months old *Lmna*^{H222P/H222P} mice. We observed an increased NOX2 protein and mRNA expressions in hearts from *Lmna*^{H222P/H222P} mice compared to wild type mice (Figure 1C, 1D). We also examined expression of the antioxidant glutathione peroxidase enzyme 1 (GPX1) involved in the detoxification of hydrogen peroxide and lipid peroxide. We showed that GPX1 expression was decreased in hearts from *Lmna*^{H222P/H222P} mice compared to wild type mice (Figure 1C). Taken together these results showed that oxidative stress was increased in *LMNA* cardiomyopathy, which is associated with reduced antioxidant protection.

N-acetyl cysteine (NAC) improves cardiac redox homeostasis in *Lmna*^{H222P/H222P} mice

Given that antioxidant defense was reduced in the heart from *Lmna*^{H222P/H222P} mice, we tested whether the antioxidant N-acetyl cysteine (NAC), a glutathione precursor, could delay the development of left ventricular dysfunction. *Lmna*^{H222P/H222P} mice were treated with NAC (140 mg/kg/day), starting at 6 months of age for 4 weeks duration (Figure 2A). We observed that the increased protein carbonylation content was decreased in hearts from

NAC-treated *Lmna*^{H222P/H222P} mice compared to vehicle-treated mice (Figure 2B). Next, we showed that the level of key antioxidant glutathione was lowered in *Lmna*^{H222P/H222P} mice compared to wild type mice, which became increased after NAC treatment, both in serum (Figure 2C) and the hearts (Figure 2D). We also studied the expression of two mRNAs encoding enzymes controlling glutathione metabolism. *Gclc* mRNA, encoding glutamate-L-cysteine ligase, a rate-limiting enzyme in glutathione synthesis, and *GsR* mRNA, encoding glutathione reductase, important for maintaining glutathione in a reduced state, were both up-regulated by 2- and 13.7-fold, respectively, in the hearts of *Lmna*^{H222P/H222P} mice (Figure 2E). This suggests an increased expression of glutathione genes as an adaptive response to counteract an increased oxidative stress in the hearts of *Lmna*^{H222P/H222P} mice. However, we cannot rule out that other mechanisms are acting in the pathogenesis of *LMNA* cardiomyopathy, including a larger alteration of the anti-oxidant signaling. Moreover, NAC treatment decreased significantly the expression of both *Gclc* and *GsR* mRNAs in the hearts of *Lmna*^{H222P/H222P} mice. These results show that NAC treatment is able to restore altered redox homeostasis in *LMNA* cardiomyopathy.

NAC improves cardiac structure and function and ameliorates fibrosis in *Lmna*^{H222P/H222P} mice

Cardiac structure and function were then assessed by echocardiography before and after 1 month of NAC treatment in different groups of mice. After the treatment, heart size and left-ventricular dilatation were reduced in NAC-treated *Lmna*^{H222P/H222P} mice compared with vehicle-treated *Lmna*^{H222P/H222P} mice (Figure 3A). Compared with the vehicle-treated *Lmna*^{H222P/H222P} mice, the NAC-treated *Lmna*^{H222P/H222P} mice had significantly decreased of left ventricular mass and left-ventricular end systolic diameter (Table 1). NAC treatment

showed a beneficial effect on fractional shortening (FS), in *Lmna*^{H222P/H222P} mice compared to vehicle-treated *Lmna*^{H222P/H222P} mice (Table 1). In addition, we observed a significant benefit of the delta FS changes from baseline (Δ FS) in NAC-treated *Lmna*^{H222P/H222P} mice compared to vehicle-treated *Lmna*^{H222P/H222P} mice (Figure 3B). Hence, treatment with NAC for 1 month delayed the development of left-ventricular dilatation and cardiac contractile dysfunction in *Lmna*^{H222P/H222P} mice. It would be of interest to assess the role of NAC treatment over a longer period of time on the cardiac function, as well as on the survival of *Lmna*^{H222P/H222P} mice.

We next hypothesized that reducing oxidative stress in the hearts of *Lmna*^{H222P/H222P} mice would reduce myocardial fibrosis. Indeed, there was a significant reduction of myocardial fibrosis in the hearts from *Lmna*^{H222P/H222P} mice treated with NAC relative to vehicle-treated *Lmna*^{H222P/H222P} mice (Figure 4A, 4B), as evidenced by Sirius Red staining of heart sections. All together, these results show that NAC treatment ameliorates cardiac structural and functional defects in *LMNA* cardiomyopathy, which is concomitant with restoring cardiac redox balance and antioxidant regulation.

DISCUSSION

Our current findings indicate that *LMNA* cardiomyopathy is associated with increased oxidative stress and antioxidant glutathione depletion. One-month oral NAC treatment, a glutathione precursor, normalized these attributes of the cardiac disease and ameliorated the worsening of cardiac dilation and contractile dysfunction in symptomatic *Lmna*^{H222P/H222P} mice. Based on our findings in heart, we need to further assess if abnormal oxidative stress is similarly involved in the pathogenesis of skeletal muscular dystrophy in the *Lmna*^{H222P/H222P} mouse model of Emery-Dreifuss muscular dystrophy, and if NAC

treatment would be beneficial for the skeletal muscle structure and function.

There is growing evidence that oxidative stress is linked to *LMNA* mutations (17,18). The precise development of oxidative stress ensuing from *LMNA* mutations still remains unknown. It has been well established that an accumulation of ROS is a direct cause of oxidative stress producing lipid peroxidation, DNA and protein oxidative damage, and the loss of cells in cardiac diseases (18-20). The implications of oxidative damage and antioxidant therapy in cardiac diseases were previously studied (21-24), but this is the first demonstration of an antioxidant therapy directed towards *LMNA* cardiomyopathy.

How oxidative stress results in cardiac tissue injury in *LMNA* cardiomyopathy is yet to be fully elucidated, but inference can be made from a number of related experimental studies. Evidence has linked lamins dysfunction with increased generation of ROS and reduced levels of antioxidant enzymes (25). Persistent ROS can oxidize selective cysteine residues in lamins, causing alterations in the structure of the nuclear lamina (26). The accumulation of ROS could play a role in the increased levels of DNA damage and the genomic instability observed in diseases related to mutation in *LMNA*. This notion is supported by the fact that treatment of cells expressing mutated lamins with NAC reduces the amount of un-repairable DNA damage (25). In the context of diseases caused by *LMNA* mutations, ROS have been linked with DNA damage (25,26). DNA double-strand breaks, induced by ROS, appear not to be repaired properly in cells expressing mutated lamins (27). Recent studies have shown that *Lmna* null cells exhibit signs of genomic instability and unrepaired DNA (27). These data indicate that A-type lamins deficiency affects the ability of cells to properly repair DNA damage and maintain genome integrity. Thus, compounds that reduce the levels of ROS in the cell could represent another strategy to reduce genomic instability in lamin-related diseases.

In conclusion, our experiments demonstrate a novel contributory mechanism for *LMNA* cardiomyopathy triggered by oxidative stress and impaired antioxidant protection. Moreover, we have shown that NAC treatment restores glutathione levels and reduces oxidative stress damage in the hearts of *Lmna*^{H222P/H222P} mice. Because NAC pharmacodynamics and absence of toxicity are well established (28), our work supports further studies on humans to begin to evaluate the therapeutic benefits of lowering oxidative stress and assessing the benefit of such therapy in cardiomyopathy. Overall, our work points toward clinically relevant NAC as a possible future treatment option for patients with *LMNA* cardiomyopathy in addition to standard therapy.

MATERIAL AND METHODS

Animals and experimental design

Experimental procedures were performed in accordance with European legislation on animal experimentation (L358-86/609/EEC). Homozygous knock-in mice carrying the *Lmna* p.H222P mutation (*Lmna*^{H222P/H222P}) were described previously (6). Six-month-old female *Lmna*^{H222P/H222P} mice were given 1-month oral N-acetyl cysteine (NAC) (Merck; 140 mg/kg/day) in drinking water and compared to sex- and age-matched vehicle-treated *Lmna*^{H222P/H222P} and wild type (WT) mice. Animals were euthanized at 7 months of age to harvest samples.

Echocardiography

Transthoracic echocardiography was performed by an investigator unaware of the mouse genotype or treatment, with an Acuson 128XP/10 ultrasound system and a 10 MHz Acuson linear probe (Mountain View). Slight anaesthesia of the mouse at room

temperature with 0.5-1% isoflurane in O₂ (Abbott Inc) was continuously adjusted to maintain heart rate.

Biochemical analyses

Detection of carbonylated proteins was performed from heart homogenates using Oxyblot protein oxidation detection kit (Chemicon International) based on immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenyl hydrazine (DNPH). Glutathione was measured in heart homogenates and serum, according to a modification of Tietze method (29) as previously described (30,31).

RNA isolation and Real-Time PCR analyses

Total RNA was extracted from frozen hearts using RNeasy Fibrous Tissue kit (QIAGEN). Concentration of total RNA was calculated using a NanoDrop ND-1000 spectrophotometer (Labtech) and RNA quality was assessed by electrophoresis. cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Mouse primer sequences used for transcriptional analyses were as follows: *mGclC* 5'-ATCCTCCAGTTCCTGCACAT-3', 5'-TGTGAATCCAGGGCCTA-3'; *mGsR* 5'-ACCACGAGGAAGACGAAATG-3', 5'-GGTGACCAGCTCCTCTGAAG-3'; *mNox2* 5'-GGCTGGGATGAATCTAGGCCAA-3', 5'-ACTGGTTTCCTGGTGAAAGAGCGG-3'. Real-time PCR was carried out on a Light Cycler (Roche Diagnostics), using the Quantitest SYBR green kit (QIAGEN) with sense and anti-sense oligonucleotides primers for the different genes. Relative levels of mRNA expression were calculated according to the Δ CT method (32).

Immunoblotting

Total proteins were isolated from mouse heart tissue in extraction buffer (Cell Signaling) with the addition of protease inhibitors (25 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM 4-[2-aminoethyl]-benzene sulfonylfluoride hydrochloride and 2 mM Na_3VO_4). The lysates were sonicated (3 pulses of 10s at 30% amplitude) to allow dissociation of protein from chromatin and solubilization. Extracts were analyzed by SDS-PAGE using a 10% gel and transferred onto nitrocellulose membranes (Invitrogen). Subsequent to washes with Tris-buffered saline containing 1% Tween 20 (TBS-T), the membranes were blocked in 5% bovine serum albumin (BSA) in TBS-T for 1 h at room temperature, then incubated with the appropriate antibody overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies for 1h at room temperature. After washing with TBS-T, the signal was revealed using Immobilon Western Chemiluminescent HorseRadish Peroxidase (HRP) Substrate (Millipore) on a G-Box system with GeneSnap software (Ozyme). Primary antibodies used were rabbit polyclonal antibodies that recognize NOX2 (Abcam), GPX1 (Cell Signaling) and GAPDH (Santa Cruz).

Histology

Hearts from mice were fixed in 4% formaldehyde for 48 h, embedded in paraffin, sectioned at 5 mm and stained with Sirius Red trichrome. Representative stained sections were photographed using a Microphot SA (Nikon) light microscope attached to a Spot RT Slide camera (Diagnostic Instruments). Images were processed using Adobe Photoshop CS (Adobe Systems).

Statistical analyses

For echocardiographic parameters, results were expressed as median [Q1;Q3]. The statistical analyses were performed by comparing the wild type animals and *Lmna*^{H222P/H222P} mice at baseline (6 months) and by comparing the WT mice to the vehicle-treated *Lmna*^{H222P/H222P} mice post-treatment (7 months) and to evaluate the NAC treatment effect by comparing the vehicle-treated *Lmna*^{H222P/H222P} mice and the NAC-treated *Lmna*^{H222P/H222P} mice at 7 months. For each analysis a normality test was performed, followed by a Student t-test (for normally distributed data) or Wilcoxon test (for not normally distributed data). For echography parameters Student t-test was performed for the heart rate and LVFS parameters and a Wilcoxon test was performed for the other parameters. In order to have a better understanding of the LVFS% progression for each mice, we analyzed the Δ FS (Δ FS=7 months LVFS – 6 months LVFS), a Student test was performed.

For mRNA expression, statistical analysis was performed in Δ Ct and the mRNA expression values normalized to the reference gene was graphically represented. The results are presented as mean \pm SEM. The glutathione concentration (serum and heart) and fibrosis parameter, results were expressed as median [Q1;Q3]. The statistical analyses were performed by comparing the wild type animals and the vehicle-treated *Lmna*^{H222P/H222P} mice and to evaluate the NAC treatment effect by comparing the vehicle-treated *Lmna*^{H222P/H222P} mice and the NAC-treated *Lmna*^{H222P/H222P} mice. For both analyses, a Student t-test was performed for the mRNA expression and glutathione concentration (heart) parameters and a Wilcoxon test was performed for glutathione concentration (serum) and fibrosis parameter.

The significance level is taken to 5%. Statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., USA).

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Conflicts of interest

Blanca Morales Rodriguez is employed by Sanofi, a global biopharmaceutical company focused on human health.

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TABLES

Table 1| Echocardiographic parameters for *Lmna*^{H222P/H222P} mice treated with NAC.

Genotype	WT	H222P	WT	H222P	H222P
Age	6	6	7	7	7
Treatment	none	none	none	vehicle-treated	NAC
n	12	39	12	19	20
Heart rate (bpm)	523.3 [509,9;536,9]	521.7 [497,9;553,0]	529.5 [499,0;579,8]	535.7 [517,2;573,4]	539.0 [517,2;556,9]
BW (g)	23 [22;24,5]	22 [21;26]	24.5 [24;25,3]	24 [22;25]	23 [20,8;26]
IVS (mm)	0.4 [0,4;0,5]	0.4 [0,4;0,5]	0.4 [0,4;0,4]	0.5 [0,4;0,5]*	0.4 [0,4;0,5]
PW (mm)	0.4 [0,3;0,4]	0.4 [0,4;0,4]	0.5 [0,4;0,5]	0.4 [0,4;0,5]	0.4 [0,4;0,45]
LVEDD (mm)	3.4 [2,9;3,5]	3.7 [3,45;3,8] ##	3.2 [3,1;3,4]	3.6 [3,4;3,95]***	3.4 [3,18;3,73]
LVESD (mm)	2.0 [1,7;2,2]	2.6 [2,3;2,9] ###	1.9 [1,9;2]	2.7 [2,4;3,1]***	2.3 [2,1;2,7]†
LVM (mg)	32.09 [26,63;43,75]	44.80 [40,45;49,25] ##	39.20 [31,22;42,49]	46.95 [44,72;54,12]**	40.45 [37,78;45,33]††
LVFS (%)	41.311 [38,49;42,94]	28.855 [24,32;33,77] ###	40.901 [38,71;43,17]	25.714 [20,55;29,18]***	32.258 [26,68;33,33] †

BW, body weight; IVS, inter ventricular septum; PW, left ventricular posterior wall; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVM, left ventricular mass LVFS, left ventricular fractional shortening; Values are median [Q1;Q3].

$p \leq 0.05$, ## $p \leq 0.01$ and ### $p \leq 0.001$: p-value obtained between wild type (WT) and *Lmna*^{H222P/H222P} (H222P) mice at 6 months of age using a two-sided Student t-test for heart rate and LVFS parameters and a two-sided Wilcoxon test for the others parameters.

* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$: p-value obtained between wild type (WT) and *Lmna*^{H222P/H222P} (H222P vehicle-treated) mice at 7 months of age using a two-sided Student t-test for heart rate parameter and a two-sided Wilcoxon test for the others parameters.

† $p \leq 0.05$, †† $p \leq 0.01$, ††† $p \leq 0.001$: p-value obtained between NAC-treated and *Lmna*^{H222P/H222P} (H222P vehicle-treated) mice at 7 months of age using a two-sided Student t-test for heart rate parameter and a two-sided Wilcoxon test for the others parameters.

FIGURES
Four figures.

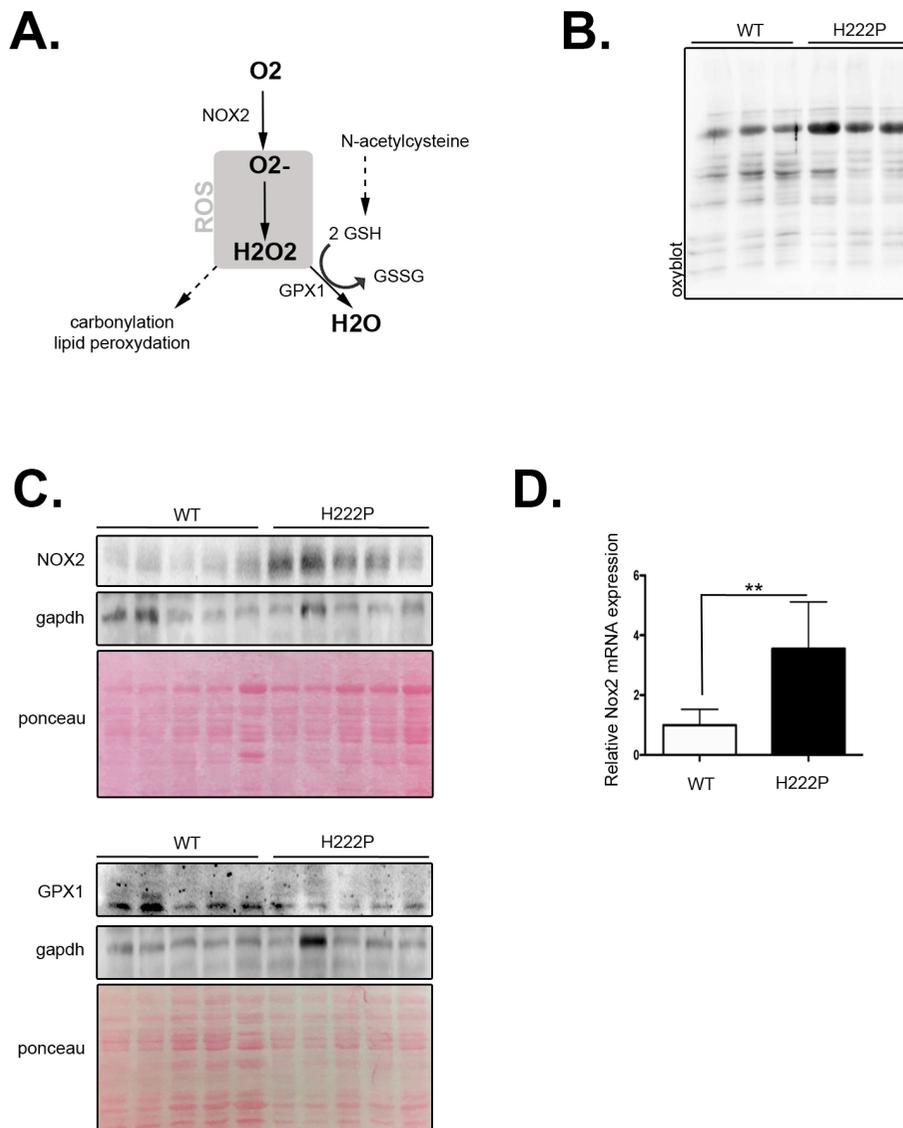
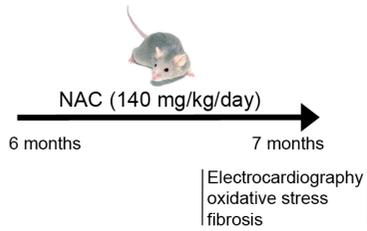


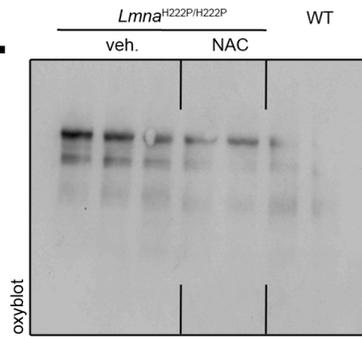
Figure 1| ROS pathway is altered in *LMNA* cardiomyopathy. (A) Schematic representation of the ROS pathway and consequences. (B) Immunoblots showing total carbonylated protein level expression (Oxyblot) in hearts from 6-months old wild type (WT) (n = 3) and from 6-months old *Lmna*^{H222P/H222P} (H222P) (n = 3) mice. (C)

Immunoblots showing NOX2, and GPX1 protein level in hearts from 6-months old wild type (WT) (n = 5) and *Lmna*^{H222P/H222P} (H222P) (n = 5) mice. Gapdh and Ponceau Red are shown as loading controls. **(D)** Expression of *Nox2* mRNA in the hearts from 6 month-old *Lmna*^{H222P/H222P} mice (H222P) (n = 5) and wild type (WT) (n = 6). Data are normally distributed and represented as means \pm SEM (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$: p-value obtained using a two-sided Student t-test on delta Ct values.).

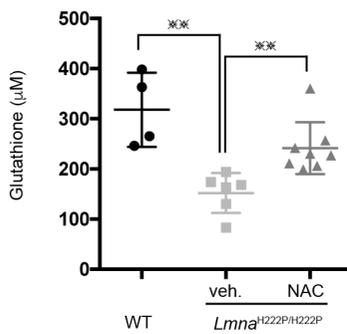
A.



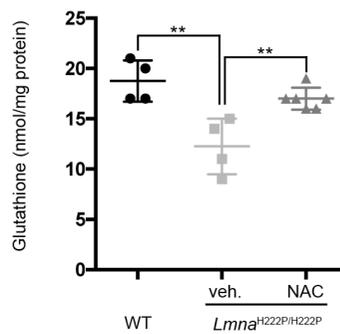
B.



C.



D.



E.

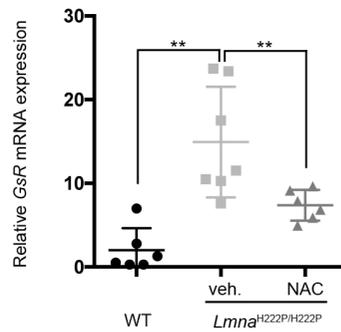
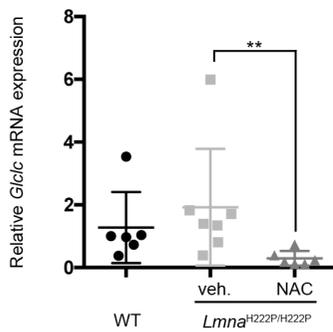
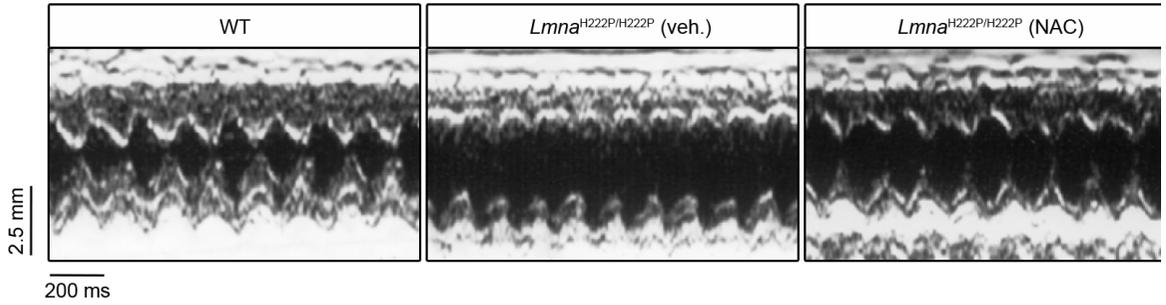


Figure 2 | N-acetyl cysteine (NAC) decreases oxidative stress in LMNA cardiomyopathy. (A) Schematic representation of the treatment protocol of *Lmna*^{H222P/H222P} (H222P) mice with N-acetyl cysteine. (B) Immunoblots showing total carbonylated protein level expression (Oxyblot) in hearts from 7-months old wild type (WT) (n = 2) and from 7-months-old *Lmna*^{H222P/H222P} (H222P) mice treated with NAC-treated (n = 2) or vehicle-treated (n = 2). Graph showing total glutathione level in sera (C) and heart (D) from 7-months-old *Lmna*^{H222P/H222P} (H222P) mice treated with NAC-treated (n = 8 in sera and n=6 in heart) or vehicle-treated (n=6 in sera and n=4 in heart). Wild type (WT) mice (n = 4 in sera and n=4 in heart) are shown as control. Data are represented as median [Q1; Q3] for figure C and as means \pm SEM for D E F. Total glutathione level in serum was not normally distributed, then a Wilcoxon test was performed for this parameter, all other parameters were normally distributed, then a Student t-test was performed for these parameters. ([□] $p \leq 0.05$, ^{□□} $p \leq 0.01$ and ^{□□□} $p \leq 0.001$: p-value obtained using a two-sided Wilcoxon test. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$: p-value obtained using a Student t-test). Quantitative real time PCR analysis of *Glclc* (E) and *GsR* mRNA expression, in 7-months-old wild type mice (WT) (n = 6), *Lmna*^{H222P/H222P} (H222P) mice treated with NAC-treated (n = 6) or vehicle-treated (n = 7). Data are represented as means \pm SEM (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$: p-value obtained using a Student t-test on delta Ct values).

A.



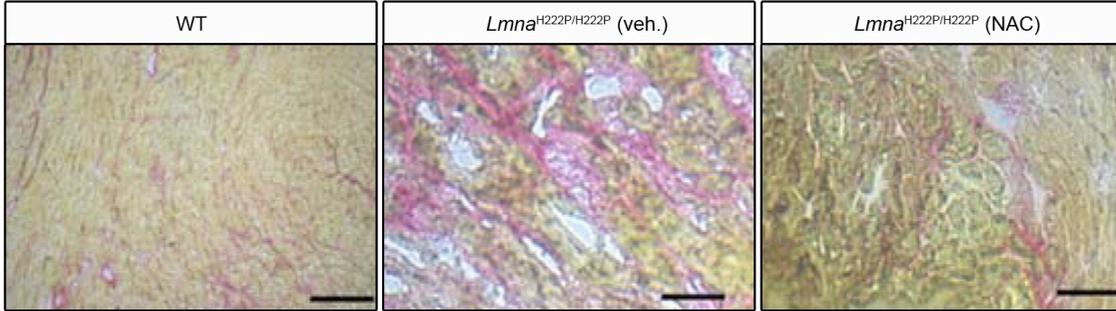
B.



Figure 3| N-acetyl cysteine improves cardiac structure and function in *LMNA*

cardiomyopathy. (A) M-mode echocardiography recording at 7 months of age in wild type (WT), vehicle-treated *Lmna*^{H222P/H222P} (H222P) and NAC-treated *Lmna*^{H222P/H222P} mice. **(B)** Fractional shortening evolution from 6 months to 7 months from wild type (WT) (n=12), vehicle-treated *Lmna*^{H222P/H222P} (H222P) (n= 19) and NAC-treated *Lmna*^{H222P/H222P} mice (n=20). Δ LVFS changes from baseline is calculated as [FS at 7 months – FS at 6 months] for each mice. Data are normally distributed and are represented as median [Q1;Q3]. (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$: p-value obtained using a two-sided Student t-test).

A.



B.

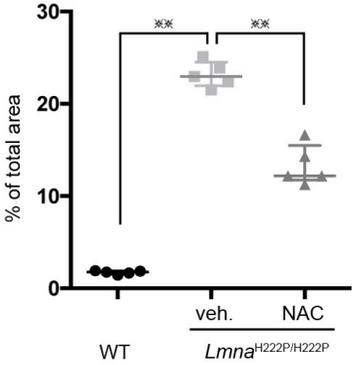


Figure 4| N-acetyl cysteine improves myocardial fibrosis in *LMNA* cardiomyopathy. (A) Sirius Red staining of cross sections of the hearts from wild type (WT), vehicle-treated *Lmna*^{H222P/H222P} (H222P) and NAC-treated *Lmna*^{H222P/H222P} mice. Scale bar: 50 μ m. (B) Heart fibrosis quantification from wild type (WT) (n=5), vehicle-treated *Lmna*^{H222P/H222P} (H222P) (n=5) and NAC-treated *Lmna*^{H222P/H222P} mice (n=5). For each mice 5 cross-section were quantified and the mean of the 5 cross-section were represented and analyzed. Data are represented as Median [Q1; Q3] ([□] $p \leq 0.05$, ^{□□} $p \leq 0.01$ and ^{□□□} $p \leq 0.001$: p-value obtained using a Wilcoxon test on mean value of the 5 cross-sections.

Activation of sarcolipin expression links mutations in A-type lamins genes to cardiomyopathy

Manuscript in preparation

Introduction

Mutations in *LMNA* encoding nuclear A-type lamins, have been associated with dilated cardiomyopathy (e.g., *LMNA* cardiomyopathy). *LMNA* cardiomyopathy is characterized by left ventricular dysfunction and dilatation often leading to heart failure. Different mechanisms contributing to the pathology had been identified, though no curative treatment has been yet found. Thus, it is essential to increase our current knowledge of the molecular mechanisms implicated in the development of *LMNA* cardiomyopathy to further identify potential therapeutic interventions. Altered calcium (Ca^{2+}) handling has been shown as playing an important role in initiation and progression of cardiomyopathies of several etiologies. Notwithstanding, we are lacking insight on the role of calcium handling in *LMNA* cardiomyopathy.

Objectives of the study

Sln gene, encoding sarcolipin, was hyperactivated in heart tissues from *Lmna*^{H222P/H222P} mice (*Muchir et al., 2007b*). Sarcolipin is an inhibitor of the sarco/endoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) and therefore may play a role in cardiac calcium handling. We decided to study the role of sarcolipin on calcium homeostasis in *LMNA* cardiomyopathy. The aim of this study was to determine the involvement sarcolipin in the progression of *LMNA* cardiomyopathy and to determine its function in intracellular Ca^{2+} handling.

Materials and methods

We analyzed the expression, localization and function of the major calcium regulators in the heart tissue of *Lmna*^{H222P/H222P} mice along the course

of the cardiac disease. We also studied the role played by sarcolipin on calcium regulators using in-house novel cellular models.

Results

We have reported an overexpression of sarcolipin that appears from early stages of the cardiomyopathy and maintained along disease progression in *Lmna*^{H222P/H222P} mice. We have also shown that expression of other calcium handling proteins (e.g. phospholamban and NCX₁) were altered in the hearts from *Lmna*^{H222P/H222P} mice. We next showed that sarcolipin over-expression was implicated in the altered expression and localization of calcium handling proteins.

Impact

We provided molecular insights of altered calcium handling in *LMNA* cardiomyopathy, encouraging further studies to mechanistically assess the role played by sarcolipin on cardiac calcium handling.

Activation of sarcolipin expression links mutations in A-type lamin gene to cardiomyopathy

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ABSTRACT

Cardiomyopathy caused by lamin A/C gene (*LMNA*) mutations (hereafter referred as *LMNA* cardiomyopathy) is an anatomic and pathologic condition associated with muscle and electrical dysfunction of the heart, often leading to heart failure-related disability. There is currently no specific therapy available for patients that target the molecular pathophysiology of *LMNA* cardiomyopathy. Searching for alternative ways to slow down the cardiac disease progression, we here examined the involvement of calcium homeostasis in the progression of the cardiac disease in a mouse model of *Lmna* cardiomyopathy. We show here that sarcolipin was abnormally elevated in the heart of the mutated mice compared with wild type mice, leading to an alteration of calcium handling. These results suggest a novel role for sarcolipin on calcium homeostasis in cardiac muscle and provide a rationale for future therapeutic interventions to *LMNA* cardiomyopathy.

Keywords: sarcolipin, calcium handling, nuclear envelope, lamin, dilated cardiomyopathy

INTRODUCTION

Dominant mutations in the gene encoding nuclear A-type lamins (*LMNA*) cause dilated cardiomyopathy with conduction system disease (*LMNA* cardiomyopathy) (1, 2). *LMNA* cardiomyopathy is characterized by conduction defects, arrhythmias, left ventricular dysfunction and dilation, often leading to heart failure-related disability (3). The management of *LMNA* cardiomyopathy consists in controlling arrhythmia and conduction defects by implantation of a defibrillator as patients are at high risk of sudden cardiac death (4). This limits the progression of heart failure, but eventually patients require heart transplantation. There are currently no effective approaches to treat *LMNA* cardiomyopathy in the clinic. Thus, it is of great importance to clarify its molecular mechanisms and to search for potential compound to provide protection against *LMNA* cardiomyopathy.

In order to study the pathophysiology of *LMNA* cardiomyopathy and to test possible therapeutics, we have developed a mouse model carrying a missense *Lmna* mutation, leading to the substitution of the histidine 222 by a proline (H222P), identified in a family with *LMNA* cardiomyopathy (5). Homozygous mutant (*Lmna*^{H222P/H222P}) mice develop cardiac left ventricular dilation and systolic dysfunction and have a reduced lifespan. We previously identified and studied multiple mechanisms that contribute, in part, to the pathophysiology of *Lmna* cardiomyopathy (6–10). All these signaling mechanisms have been targeted with small-molecules in *Lmna*^{H222P/H222P} mice and all of these interventions have had some beneficial impact. However, targeting each of these pathways alone is not curative. Searching for alternative ways to slow down the cardiac disease progression, we here examined the involvement of calcium homeostasis in the progression of the cardiac disease in *Lmna*^{H222P/H222P} mice. The pathophysiology of cardiomyopathy convincingly converges to the conclusion that unhinged calcium (Ca²⁺) handling in the cardiomyocytes plays a central role in initiation and progression of the disease (11). The delicate and precise regulation of Ca²⁺ cycling is finely tuned by numerous macromolecular proteins and regulatory processes, and is severely deranged in cardiomyopathy. Therefore, the mediator and/or regulatory components of the Ca²⁺ cycling apparatus have been the focus of extensive research involving targeted pharmacologic and gene interventions aiming to a restoration of Ca²⁺ cycling processes, thus improving cardiac function.

RESULTS

Activation of cardiac sarcolipin expression in *Lmna*^{H222P/H222P} mice | To explore the role of calcium signaling in the development of dilated cardiomyopathy, we studied *Lmna*^{H222P/H222P} mice, which develop dilated a progressive contractile dysfunction that recapitulates human *LMNA* cardiomyopathy (5). *Lmna* p.H222P corresponds to a human disease-causing mutation associated with dilated cardiomyopathy (12). We previously examined differential expression of mRNAs isolated from hearts of 10 week old wild type and *Lmna*^{H222P/H222P} male mice (6). This analysis revealed activation of the expression of *Sln* gene, encoding sarcolipin, in hearts of the *Lmna*^{H222P/H222P} mice compared with wild type mice (6) (**Figure 1A**). The up-regulation of sarcolipin was observed at 24 weeks of age at both mRNA and protein levels (**Figure 1B, 1C, 1D**). We next evaluated the cardiac localization of sarcolipin in ventricular isolated cardiomyocytes. The cardiac localization of sarcolipin was not different in *Lmna*^{H222P/H222P} cells compared with wild type cells (**Figure 1E**). However, we note an increased staining of sarcolipin in mutated cells compared with wild type cells (**Figure 1E**).

Altered calcium homeostasis in *Lmna*^{H222P/H222P} mice caused by sarcolipin up-regulation | Given that sarcolipin is a regulator of cardiac calcium handling through its inhibitory role on sarcoplasmic/endoplasmic reticulum Ca^{2+} transport ATPase (Serca2a), we next hypothesized that the altered expression of sarcolipin could alter cardiac calcium homeostasis. Ca^{2+} images in *Lmna*^{H222P/H222P} ventricular myocytes showed decreased calcium transient decay (Tau) compared with wild-type cells (**Figure 2A**), while no significant difference was observed for calcium transient amplitude (F/F_0) (**Figure 2B**). Because several proteins tightly regulate defective $[\text{Ca}^{2+}]_{\text{intracellular}}$ cycling, we investigated whether the expression of calcium handling proteins was altered in *Lmna* cardiomyopathy. We found that expression of both Serca2a and Ncx1, the two major players of $[\text{Ca}^{2+}]_{\text{intracellular}}$ removal from cardiac cells, was unaffected in hearts of the *Lmna*^{H222P/H222P} mice, at 12 weeks of age when no cardiac symptoms were detectable (**Figure 2C-D**). However, we observed an altered localization of Serca2a, but not Ncx1, in *Lmna*^{H222P/H222P} cardiac cells compared with wild type cells (**Figure 2E**). We next studied the expression of calcium handling proteins at a later stage of the disease, when left-

ventricular function was altered (**Supplemental Figure 1**). The expression of NCX1 but not Serca2a was increased in hearts of 24 weeks old *Lmna*^{H222P/H222P} mice compared with wild type mice (**Figure 3A-B**). The expression of phospholamban, another regulator of Serca2a, was decreased in the hearts of the *Lmna*^{H222P/H222P} mice (**Figure 3C-D**). This reduction is ensuing a decreased phosphorylation of phospholamban on residues Ser16 and Thr17 in the hearts from *Lmna*^{H222P/H222P} mice (**Figure 3B**).

To dissect the molecular mechanism(s) responsible for sarcolipin activation and altered calcium homeostasis, we used cell culture models. We used lentiviral transduction to generate cardiac cells (HL-1 and H9C2) stably expressing FLAG epitope-tagged sarcolipin (**Figure 4A, 4B**). We confirmed that expression of Serca2a was not changed in HL-1 and H9C2 cells expressing FLAG epitope-tagged sarcolipin, compared to untransduced cells (**Figure 5A, 5B**). *Ncx1* expression was activated only in H9C2 stably expressing FLAG epitope-tagged sarcolipin (**Figure 5A, 5B**). The expression of total phospholamban as well as its phosphorylation on Ser16 was decreased in HL-1 but not H9C2 cells expressing FLAG epitope-tagged sarcolipin (**Figure 5A, 5B**). The localization of phospholamban was altered in HL-1 and H9C2 cells expressing FLAG epitope-tagged compared untransduced cells (**Figure 6A, 6B**). All together, these results suggest that abnormal calcium homeostasis caused by sarcolipin overexpression may be a contributor of the development of *LMNA* cardiomyopathy.

Reducing sarcolipin expression does not mitigate cardiac dysfunction in *LMNA* cardiomyopathy

We next tested the hypothesis that the down-regulation of sarcolipin could influence left ventricular function *in vivo*. We injected adeno-associated virus (AAV) vectors expressing either a short hairpin RNA against sarcolipin tagged-GFP (*shSln*) or empty AAV (null) into 6 weeks old *Lmna*^{H222P/H222P} mice. The *shSln* treatment for 18 weeks significantly reduced sarcolipin expression (**Figure 7A-B**) ($p < 0.05$) in cardiac muscle of the *shSln* treated mice compared to the null treated mice. Expression of total phospholamban was unchanged by downregulating sarcolipin (**Figure 7A-B**). To determine the cardiac transduction level of *shSln* we performed immunostaining against the GFP-tag. 20% of the cardiac cells were transduced (**Figure 7C**). We next evaluated the effect of the reduction of sarcolipin on the cardiac function. No significant effects of the reduction of sarcolipin expression were observed on cardiac chambers dimensions nor left

ventricular function (**Figure 7C**). All together, these results suggest that a partial reduction of sarcolipin has no therapeutic effect on *Lmna* cardiomyopathy.

DISCUSSION

In this study, we have shown that sarcolipin expression is up regulated early along the progression of cardiomyopathy in heart from *Lmna*^{H222P/H222P} mice. The increased expression of sarcolipin is accompanied by alteration of calcium handling proteins (e.g. phospholamban and Ncx1). These results suggest a novel model for A-type lamins in the regulation of calcium homeostasis and pathophysiology. In this model, activation of sarcolipin inhibits Serca2a and blunt $[Ca^{2+}]_{intracellular}$ removal from the cytosol to the sarcoplasmic reticulum. Therefore, our work encourages further approaches to mechanistically assess the role played by sarcolipin on calcium handling.

Ca^{2+} is a mediator of excitation contraction coupling, and compounds that target calcium handling may lead to improved striated muscle function. Overexpression of SERCA improves the outcome in muscular dystrophy (13), and increasing Ca^{2+} influx into muscle is sufficient to produce muscular dystrophy (14). These findings suggest that drugs that could correct impaired calcium handling could ameliorate left ventricular dysfunction in *LMNA* cardiomyopathy. Similar pathogenic mechanism of sarcolipin mediated modulation of calcium handling may play a role in left ventricular dysfunction in other forms of cardiomyopathy, in which there appears to be abnormal inhibition of SERCA2a activity (15–18). The overexpression of sarcolipin has been detected in humans with different cardiac pathologies contributing to the contractile dysfunction (19, 20). Therefore, we could speculate that the regulation of sarcolipin may not be specific to *LMNA* cardiomyopathy but occurs in several cardiomyopathies.

Scant studies focused on aberrant regulation of intracellular calcium in *Lmna* cardiomyopathy. Arimura and colleagues showed that the use of pyridazinone derivative calcium (Ca^{2+}) sensitizing agent SCH00013 was beneficial on cardiac function in *Lmna*^{H222P/H222P} mice, inducing an increased life expectancy and a decreased fibrosis (21). In this study, we have reported for the first time a delay on calcium recapture by the sarcoplasmic reticulum in cardiomyocytes from *Lmna*^{H222P/H222P} mice. This delay can be explained either by an altered Serca2a activity or a Ncx1 dysfunction (22, 23). As observed in sarcolipin overexpression rodent models (24, 25), the cardiomyocytes contractility was

impaired and the relaxation was delayed. Then, the regulation of SERCA2a by targeting sarcolipin expression in *LMNA* cardiomyopathy could be an interesting approach to re-establish normal Ca^{2+} homeostasis. Recent studies on Duchene muscular dystrophy, in which cardiac phenotype is similar to the one observed in *LMNA* cardiomyopathy (26), reported a cardiac sarcolipin overexpression in *mdx:utr^{-/-}* mice (27).

In conclusion, we showed for the first time calcium-handling disturbance in *Lmna* cardiomyopathy. We also showed that sarcolipin activation and Serca2a inactivation could be participating to the pathogenesis of *Lmna* cardiomyopathy. The study has several shortcomings. Whereas sarcolipin expression was decreased in sh*Sln*-injected *Lmna*^{H222P/H222P} mice, cardiac function, as assessed by echocardiography, was not improved. Voit *et al* performed an AAV-mediated therapy against sarcolipin and observed an amelioration of the cardiac phenotype and an increase of the lifespan of *mdx:utr^{-/-}* mice (27). However, our findings suggest that a 40% reduction of sarcolipin expression is not enough to ameliorate cardiac function. Therefore, we could speculate that the low transduction level was not enough to impact the Serca2a function. Thus it will be of great interest to increase the efficiency of the treatment and to analyse its effect on the Serca2a pump in order to determine the therapeutic potential of this inhibition. In addition to heart failure, cardiac conduction defects are also important causes of death in *LMNA* cardiomyopathy patients. Telemetric cardiac rhythm monitoring and electrophysiological studies were not performed in sh*Sln*-injected *Lmna*^{H222P/H222P} mice, partly because of the small group size of treated mice, to determine contributions of cardiac conduction defects to premature death and improved survival in the experimental groups.

In conclusion, these findings implicate *Lmna* in the regulation of calcium handling. Dysregulations of sarcolipin and Serca2a function collectively, are in part responsible for induction of cardiac phenotype in the disease. The findings identify sarcolipin as potential target to prevent or attenuate cardiac phenotype in *LMNA* cardiomyopathy.

MATERIAL AND METHODS

Mice | All animal experiments were performed in accordance with guidelines from directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Wild type and *Lmna*^{H222P/H222P} mice were fed ad libitum housed in a disease-free barrier facility with 12h/12h light/dark cycles.

Isolation of mouse cardiomyocytes | Mice were anesthetized with 2-3% isoflurane and placed in 37°C heating pad. The heart was removed and the aorta cannulated. After washing buffer was perfused for three minutes, 1mg/ml Liberase (Liberase Research Grade, Roche) solution was perfused through the coronary arteries for 6min with 2,3-Butanedione monoxime (BDM) 10mM and $[Ca^{2+}] = 0.1mM$. Ventricular tissue was teased apart and pipetted to release individual cells. After enzymatic dispersion, Ca^{2+} concentration in the buffer containing 2.7mg/ml bovine serum albumin was elevated in three steps up to 1.8mM.

Cell culture and reagents | H9C2 (gift from Katarzyna Piekarowicz) and HL-1 (Merck Millipore) were maintained at 37 °C with 5% CO₂ and subcultured at 80–90% confluency. Unmodified and stable H9C2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Unmodified and stable HL-1 cells were culture in Claycomb Medium (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Invitrogen), norepinephrine 0.1mM (Sigma-Aldrich) in 30mM ascorbic acid (Sigma-Aldrich) and L-glutamine 2mM with the required gelatin/fibronectin coating. H9C2 and HL-1 expressing FLAG epitope-tagged sarcolipin were created using lentiviral vector EF1.GOI (Sarcolipin Tag Flag).WPRE (Vectalys). MOI: 100, cells were incubated with the lentiviral vector for 24h followed by a 2-day incubation with normal medium and a 3-5 days puromycin selection. After selection H9C2 and HL-1 expressing FLAG epitope-tagged sarcolipin were maintained in their normal culture conditions.

RNA isolation and real-time PCR | Total RNA was extracted from nitrogen-frozen heart using RNAeasy Fibrous Tissue kit (QIAGEN). 600ng of total RNA measured using a NanoDrop ND-1000 spectrometer (Labtech) was subjected to cDNA synthesis using the SuperScript III First-Strand Synthesis for RT-PCR (Invitrogen). Mouse primer sequences used for transcriptional analyses were as follows: *mSln* 5'-gtccttgtagcctgagtgt-3', 5'-acggatgatgaggacaactgt-3'; *mRplp0* 3'-atagccttgcgcatcatggt-5', 3'-ctccaagcagatgcagcaga-5'. Real-time PCR was performed on Light Cycler 480 (Roche Diagnostics), using SYBR green PCR Master Mix (Applied Biosystems). PCR products were subjected to melting curve analysis to exclude the synthesis of non-specific products. Cycle threshold (C_t) values were quantified using a standard curve for the specific gene and relatively quantified using *RplR0* as an internal reference control. The C_t values were then normalized to the average expression levels of samples, calculated according to the ΔC_t method (28) and are presented as fold change over wild type controls. All experiments were performed in triplicates.

Protein extraction and western blot analysis | Total proteins were isolated from heart tissue or cultured cell in extraction buffer (Cell Signaling). The heart samples were extracted with Lysis-D tube using Fast-Prep (3 pulses of 45 s), the cells samples were sonicated (5 pulses of 10s at 30% amplitude) to allow dissociation of protein from chromatin and solubilization. Sample protein content was determined by the BiCinchoninic Acid Assay protein assay (Thermo Fisher Scientific). Extracts were analysed by SDS-PAGE using either 4-12% or 4-20% (for sarcolipin, phospholamban and FLAG) Tris-glycine gels (Life Technologies) and transferred onto nitrocellulose 0.2 μ m membrane (novex, Life Technologies). Membranes were blocked one hour with 5% bovine serum albumin (BSA) in phosphate buffer saline containing 1% Tween 20 (PBS-T). Subsequently to blocking, the membranes were incubated with primary antibodies overnight at 4°C. After TBS-T washes membranes were incubated with anti-chicken, anti-mouse or anti-rabbit antibodies for 1h at room temperature and washed again with TBS-T. The signal was then revealed using Immobilon Western Chemiluminescent HorseRadish Peroxidase (HRP) Substrate (Millipore) on a G-Box system with GeneSnap software (Ozyme).

Antibodies | Primary antibodies used were: sarcolipin for western blot (Merck Millipore, #ABT13) and for immunofluorescence (Proteintech, #18395-1-AP), NCX1 (Abcam, #ab2869), SERCA2a (Abcam, #ab2861), phospholamban (Cell Signaling, #14562), phospho-phospholamban(Ser16) (Santa Cruz, SC-12963-R), phospho-phospholamban(Thr17) (SC-17024-R Santa Cruz), FLAG (F3165 Sigma), GFP (A10262 Invitrogen), α -actinin (A7732 Sigma), GAPDH (sc-47724 Santa Cruz). Secondary antibodies for immunoblotting were HRP-conjugated donkey anti-chicken (Invitrogen), rabbit anti-mouse or goat anti-rabbit IgG (Jackson ImmunoResearch). Secondary antibodies for immunofluorescence were Alexa Fluor 488 conjugated goat anti-rabbit IgG, Alexa Fluor 568-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated goat anti-chicken IgG (Life Technologies).

Immunofluorescence microscopy | For immunofluorescence microscopy, isopentane-frozen hearts were cut into 8 μ m-thick sections. Cryosections were fixed (15min, 4% paraformaldehyde in PBS at room temperature), permeabilized (10min, 0,5% Triton X-100 in PBS), blocked (1h30, in PBS with 0,3% triton X-100 and 5% BSA) and incubated with primary antibodies (overnight at 4°C, in PBS with 1% triton X-100 and 1% BSA). The section were then washed in PBS (3 times 5 min) and incubated with secondary antibodies (1h at RT, in PBS with 1% triton X-100 and 1% BSA) and washed (3 times 5 min).

HL-1, HL-1 expressing FLAG epitope-tagged sarcolipin were grown in coated (gelatin/fibronectin) coverslips and H9C2, H9C2 expressing FLAG epitope-tagged sarcolipin in normal coverslips. Cells were fixed (10 min, 4% paraformaldehyde in PBS at room temperature), permeabilized (8 min, 0,5% Triton X-100 in PBS), blocked (1h, in PBS with 0,3% triton X-100 and 5% BSA) and incubated with primary antibodies (1h30 at RT, in PBS with 1% triton X-100 and 1% BSA). The section were then washed in PBS (3 times 5 min) and incubated with secondary antibodies (1h at RT, in PBS with 1% triton X-100 and 1% BSA) and washed (3 times 5 min).

Echocardiography | Mice were lightly anesthetized with 0,5-1% isoflurane and placed in a heating pad. Transthoracic echocardiography was performed using an ACUSON 128XP/10 ultrasound with an 11 MHz transducer. All parameters were measured by a “blinded

echocardiographer” unaware of the genotype or treatment of the mice, performed in 2D mode and M-mode in triplicate.

Construction of plasmids encoding FLAG epitope-tagged Sarcolipin WT |

Sarcolipin plasmid (MC204674 OriGene Technologies) was used as a template. FLAG epitope was added to the sarcolipin sequence by PCR using the following primers (Flag: F-5'cgaagcttgcccaccatggattacaaggacgacgatgacaagatggag 3', R-5'-cccagcggccgccctcagtatt-3'). Then the the sarcolipin sequence was cloned in a pGG2 vector (gift from Frédérique Rau) using (Hind III and Not I). The final plasmid was transformed in XL-10 (Agilent).

Construction of lentivirus encoding for FLAG epitope-tagged Sarcolipin| FLAG epitope-tagged Sarcolipin cassette was cloned into the lentiviral expression plasmid pLV.EF1.IRES2.Puromycine using HindIII and NcoI restriction enzymes by Vectalys company.

Construction and injection of AAV

AAV9-GFP-U6-m*Sln*-shRNA and AAV-U6-null (VectorBiolabs) Recombinant AAV viruses are produced through co-transfecting HEK293 cells with AAV plasmid with sh-*Sln*, plus other needed helper-plasmid DNAs. In the AAV plasmid, the REP and CAP genes of wild type AAV were deleted, so there were only 2 copies of ITRs (~145 bp/each) left. 2 days after transfections, cell pellets were harvested, and viruses were released through 3x cycles of freeze/thaw. Viruses were purified through CsCl-gradient ultracentrifugation, followed by desalting. Viral titer (GC/ml - genome copies/ml) was determined through real-time PCR. AAV9-GFP-U6-m*Sln*-shRNA and AAV9-U6-null were injected at 5×10^{13} vg/kg at 6 weeks of age by retro-orbital injection.

Statistics| Statistical analyses were performed using GraphPad Prism software.

For echocardiographic parameters, results were expressed as median [Q1;Q3]. The statistical analyses were performed by comparing the wild type (WT) animals and

Lmna^{H222P/H222P} mice at 12 and 24 weeks or by comparing *Lmna*^{H222P/H222P} treated with an

AAV9-null and *Lmna*^{H222P/H222P} treated with an AAV9-sh*Sln* -GFP. For each analysis (LVFS%, LVEDS, LVEDD parameters) a Wilcoxon test was performed for. For mRNA expression, statistical analysis was performed in Δ Ct and the mRNA expression values normalized to the reference gene (*Rplp0*) was graphically represented. The results are presented as median [Q1;Q3], , a Wilcoxon test was performed.

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Conflicts of interest |

Blanca Morales Rodriguez is employed by Sanofi, a global biopharmaceutical company focused on human health.

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FIGURES

Seven figures.

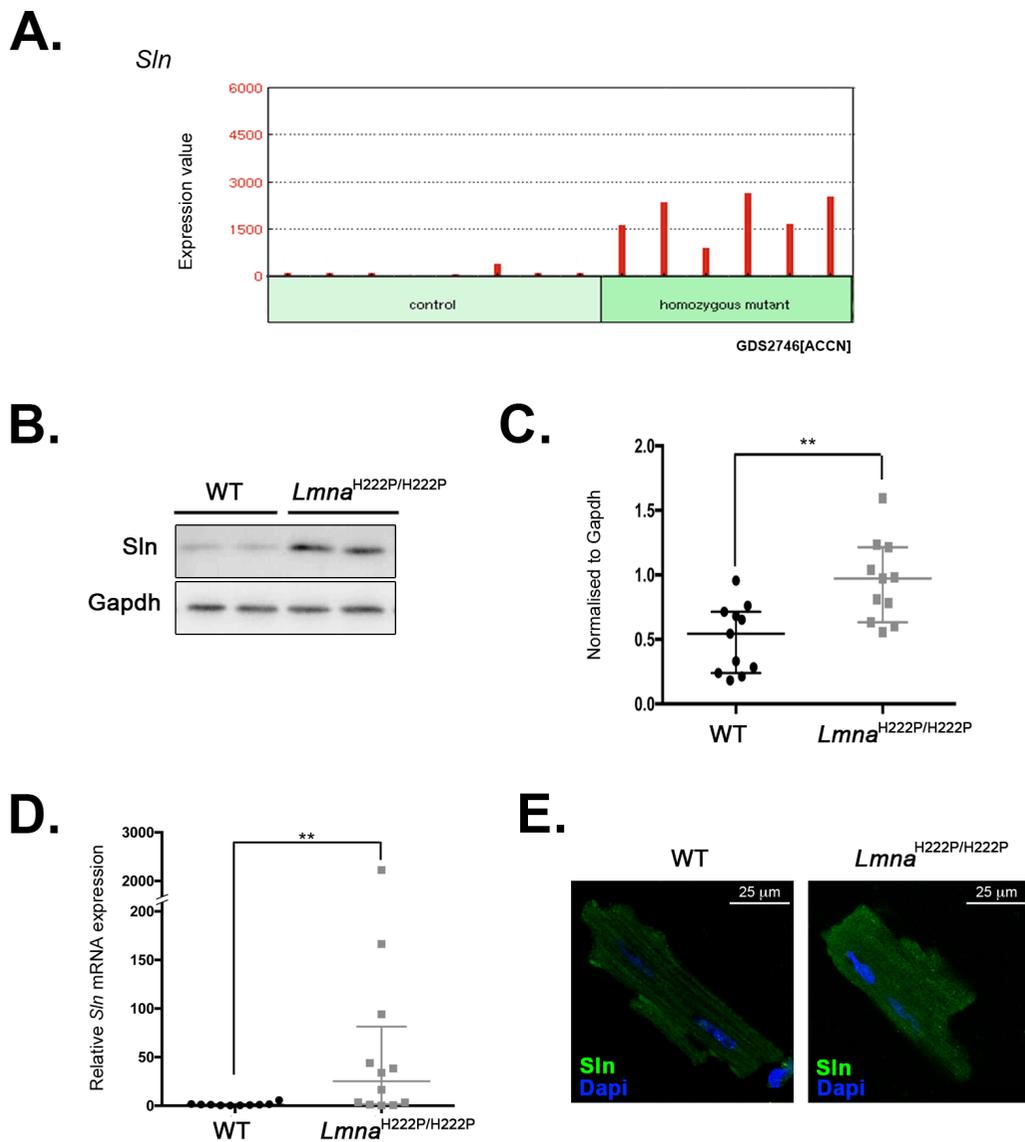


Figure 1. Activation of sarcolipin in *LMNA* cardiomyopathy | (A) Graph showing expression profile of *Sln* mRNA from Affymetrix GeneChip data between wild type (WT) (n = 8) and *Lmna*^{H222P/H222P} mice (n = 6). (B) Representative immunoblot showing sarcolipin expression in hearts from 24 weeks old male *Lmna*^{H222P/H222P} and wild type (WT) mice. Gapdh was shown as loading control. (C) Immunoblot quantification of sarcolipin expression in wild type (WT) (n = 11) and *Lmna*^{H222P/H222P} mice (n=11), normalized to

Gapdh. ** $p \leq 0.01$: p-value obtained using a student test. **(D)** Expression of *Sln* mRNA in hearts from 24 weeks old wild type (WT) (n = 12) and *Lmna*^{H222P/H222P} mice (n = 12). Data are represented as median [Q1; Q3] ** $p \leq 0.01$: p-value obtained using a Wilcoxon test on delta Ct values). **(E)** Micrographs showing sarcolipin labeling in isolated cardiomyocytes from 12 weeks old wild type (WT) and *Lmna*^{H222P/H222P} mice. Nuclei are counter-stained with 4',6-diamidino-2-phenylindole (dapi).

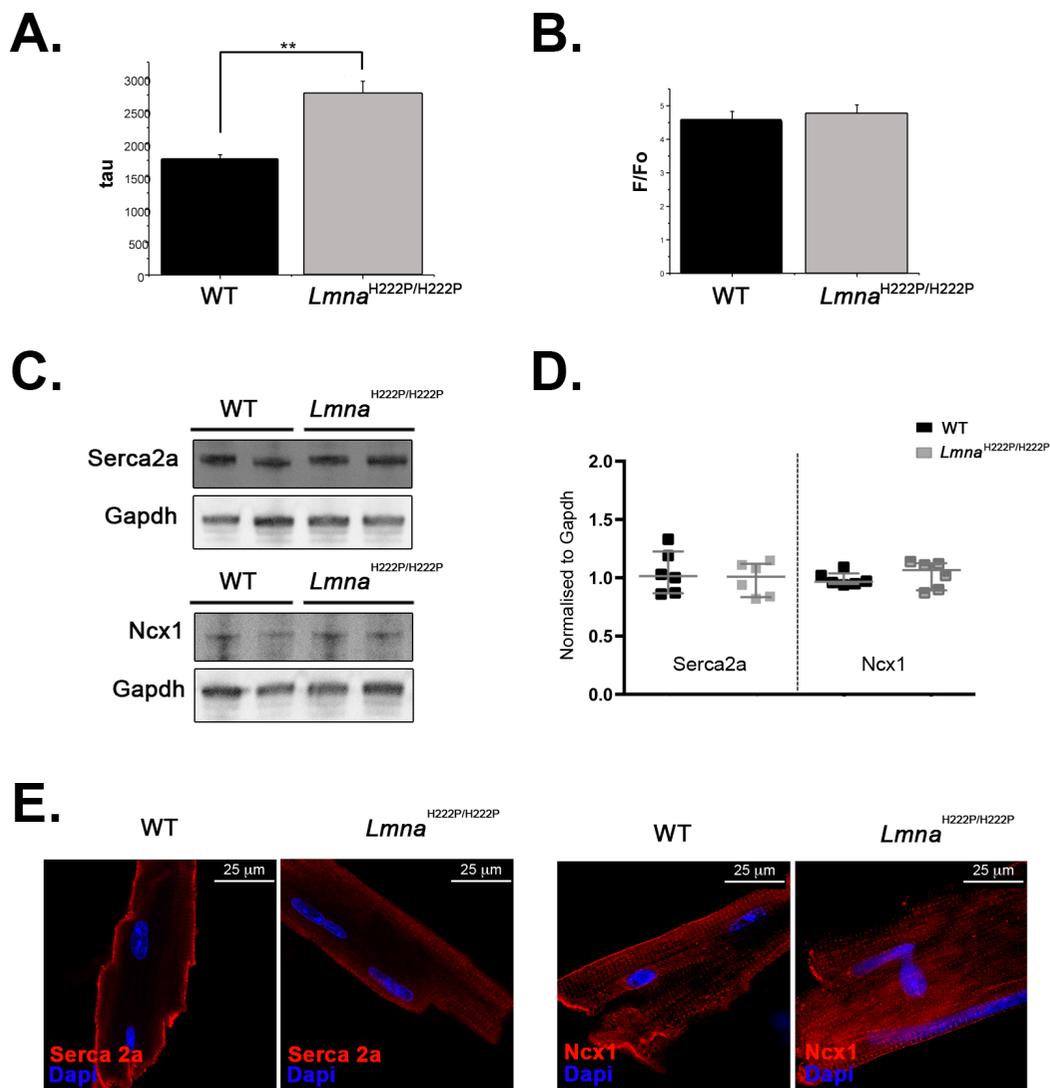


Figure 2. Calcium handling proteins in *Lmna* cardiomyopathy when no cardiac dysfunction was observed (A) Graph showing relaxation time constant (Tau) from isolated cardiomyocytes from 12 weeks old wild type (WT) and *Lmna*^{H222P/H222P} mice. Data are represented as means ± standard errors of means. ***P*<0.001. (B) Graph showing calcium transient amplitude (F/F₀) from isolated cardiomyocytes from 12 weeks old wild type (WT) and *Lmna*^{H222P/H222P} mice. Data are represented as means ± standard errors of means. (C) Representative immunoblots showing Serca2a and Ncx1 expression in hearts from 12 weeks old male *Lmna*^{H222P/H222P} and wild type (WT) mice. Gapdh was shown as

loading control. **(D)** Immunoblot quantification of Ncx1 and Serca2a expression in wild type (WT) (n = 6) and *Lmna*^{H222P/H222P} mice (n=6), normalized to Gapdh. **(E)** Micrographs showing Serca2a and Ncx1 labeling in isolated cardiomyocytes from wild type (WT) and *Lmna*^{H222P/H222P} mice. Nuclei are counter-stained with 4',6-diamidino-2-phenylindole (dapi).

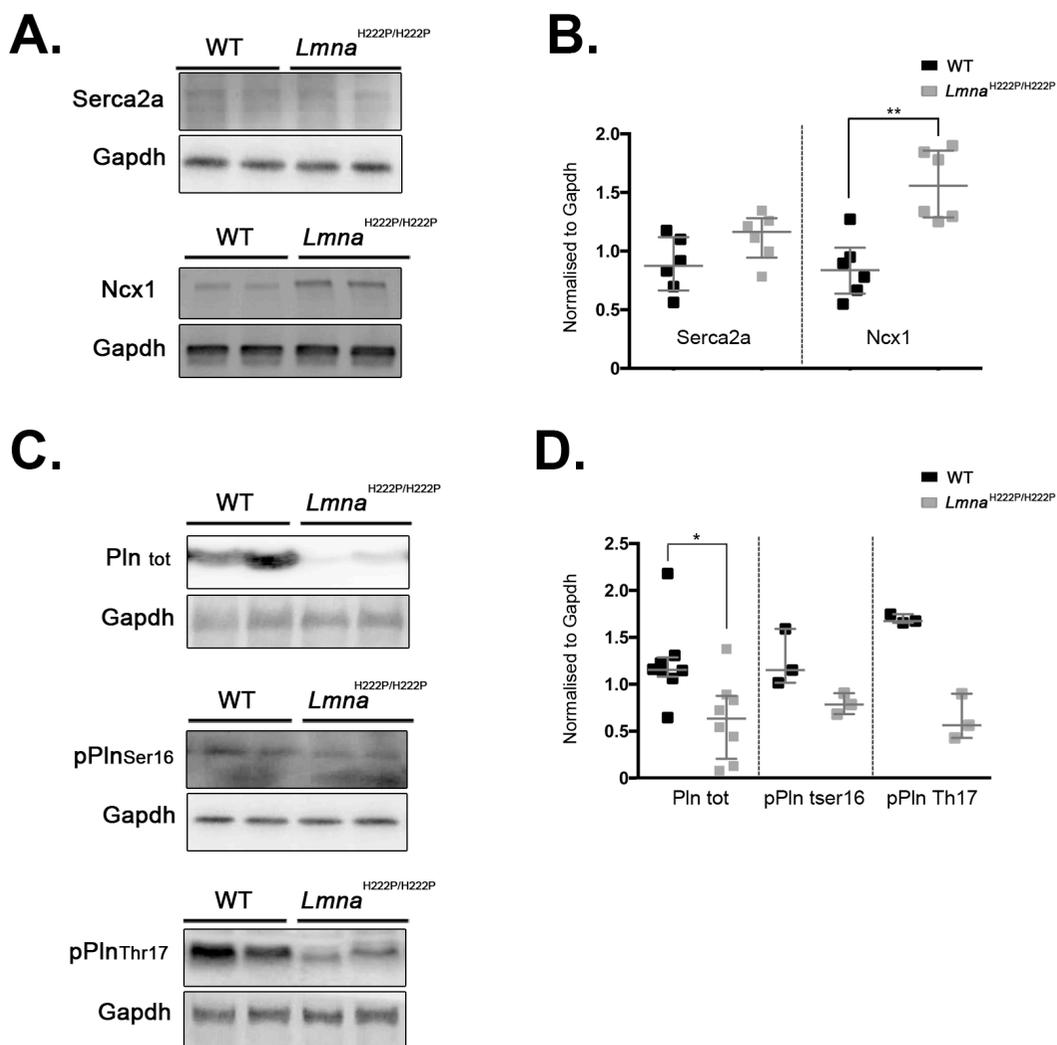


Figure 3. Calcium handling proteins in *Lmna* cardiomyopathy at symptomatic stage

(A) Representative immunoblots showing Serca2a and Ncx1 expression in hearts from 24 weeks old male *Lmna*^{H222P/H222P} and wild type (WT) mice. Gapdh was shown as loading control. (B) Immunoblot quantification of Serca2a and Ncx1 expression in wild type (WT) (n = 6) and *Lmna*^{H222P/H222P} mice (n=6), normalized to Gapdh. (C) Representative immunoblots showing total, Thr17 and Ser16 phospholamban expression in hearts from 24 weeks old male *Lmna*^{H222P/H222P} and wild type (WT) mice. Gapdh was shown as loading control. (D) Immunoblot quantification of total, Thr17 and Ser16 phospholamban

expression in hearts from 24 weeks old male *Lmna*^{H222P/H222P} and wild type (WT) mice expression in wild type (WT) (n = 6) and *Lmna*^{H222P/H222P} mice (n=6), normalized to Gapdh. *p≤0.05 : p-value obtained using a Wilcoxon test.

Morales Rodriguez et al. Figure 4

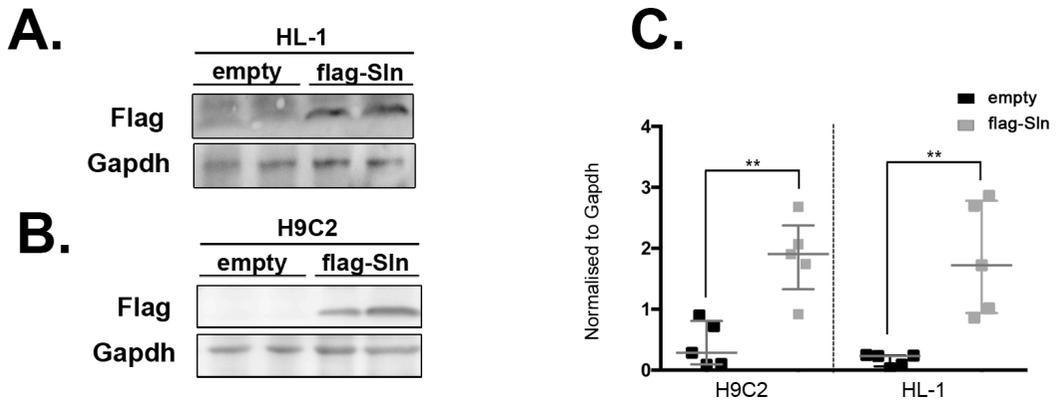
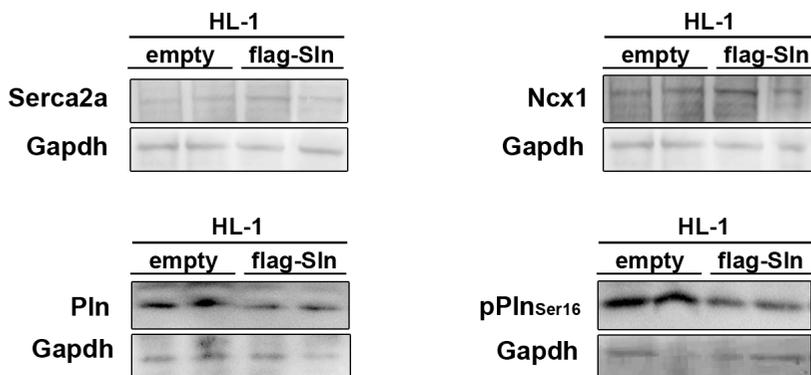


Figure 4. Cardiac cell lines stably expressing sarcolipin (A) Representative immunoblots showing FLAG expression in HL-1 cells (empty) and HL-1 cells stably expressing FLAG epitope-tagged sarcolipin (flag-Sln) (B) Representative immunoblots showing FLAG expression in H9C2 cells (empty) and H9C2 cells stably expressing FLAG epitope-tagged sarcolipin (flag-Sln). (C) Immunoblot quantification of FLAG expression in HL-1 cells, H9C2 (empty) and HL-1 and H9C2 cells stably expressing FLAG epitope-tagged sarcolipin (flag-Sln) (n=5 in all groups), normalized to Gapdh. Data are represented as median [Q1; Q3] **p≤0.01 : p-value obtained using a Wilcoxon test.

A.



B.

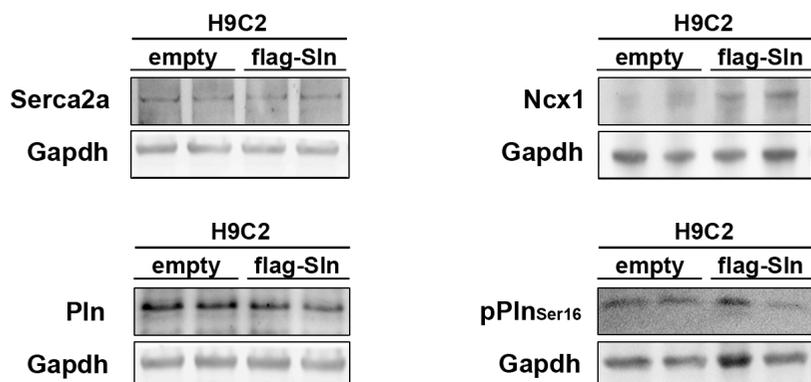
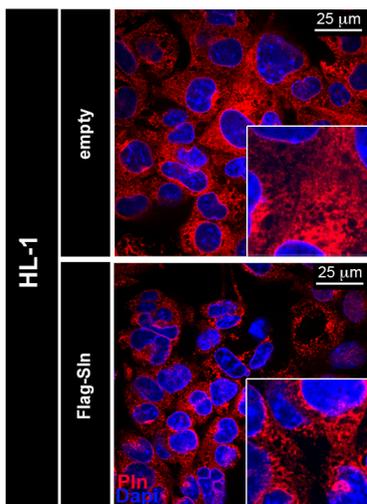


Figure 5. Alteration of calcium handling regulators in cardiac cell lines stably expressing sarcolipin| (A) Representative immunoblots showing Serca2a Ncx1, total phospholamban and phosphorylated phospholamban (Ser₁₆) expressions in HL-1 cells (empty) and HL-1 cells stably expressing FLAG epitope-tagged sarcolipin (flag-Sln) **(B)** Representative immunoblots showing Serca2a Ncx1, total phospholamban and phosphorylated phospholamban(Ser₁₆) expressions in H9C2 cells (empty) and H9C2 cells stably expressing FLAG epitope-tagged sarcolipin (flag-Sln).

A.



B.

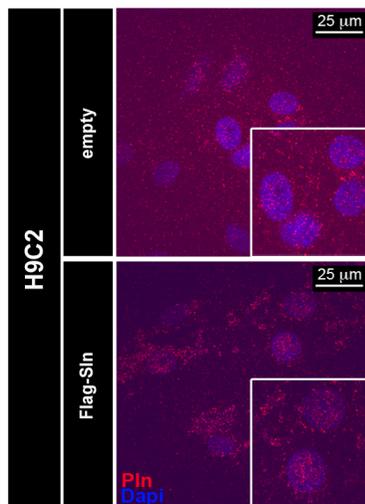


Figure 6. Localization of phospholamban in cardiac cell lines stably expressing sarcolipin (A) Micrographs showing phospholamban (PIn) labeling in HL-1 cells (empty) and HL-1 cells stably expressing FLAG epitope-tagged sarcolipin (flag-Sln). Nuclei are counter-stained with 4',6-diamidino-2-phenylindole (dapi). (B) Micrographs showing phospholamban (PIn) labeling in H9C2 cells (empty) and H9C2 cells stably expressing FLAG epitope-tagged sarcolipin (flag-Sln). Nuclei are counter-stained with 4',6-diamidino-2-phenylindole (dapi).

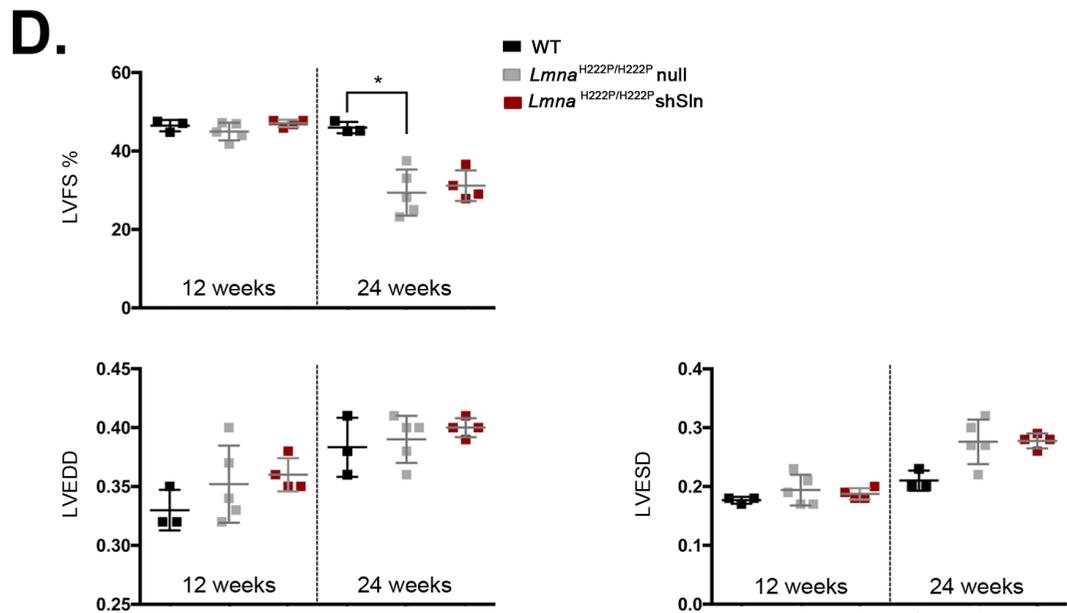
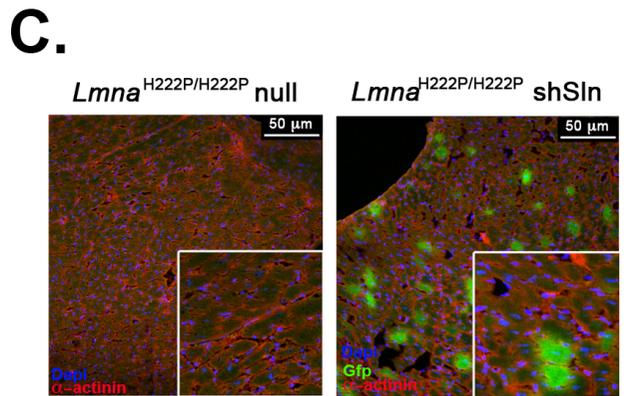
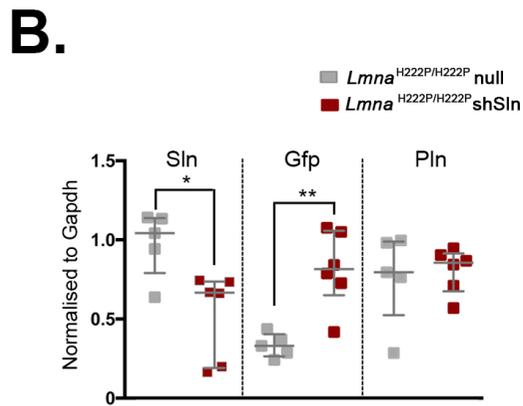
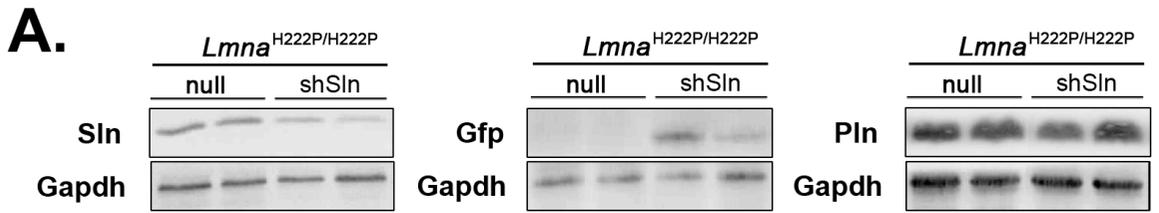
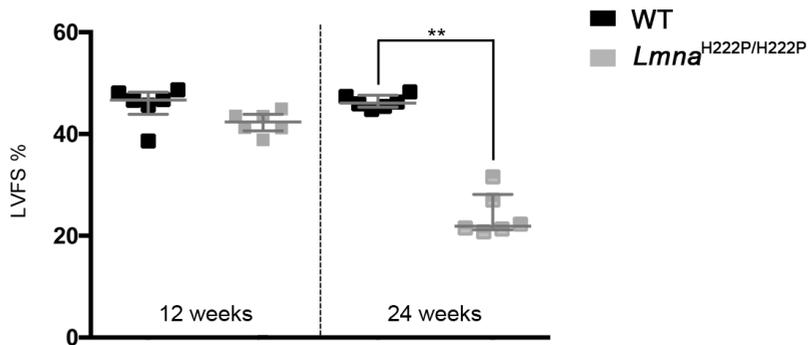
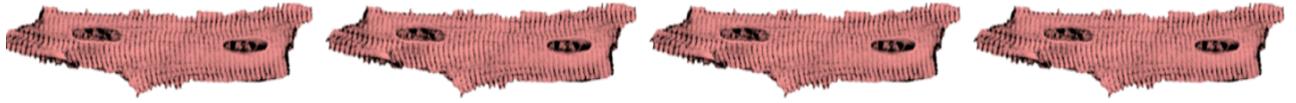


Figure 7. AAV9-sh*Sln*-GFP treatment (A) Representative immunoblots showing *Sln*, *Gfp* and total phospholamban expressions in hearts from 24 weeks old male *Lmna*^{H222P/H222P} treated with an AAV9-null (null) and *Lmna*^{H222P/H222P} treated with an AAV9-sh*Sln*-GFP (sh*Sln*). *Gapdh* was shown as loading control. (B) Immunoblot quantification of sarcolipin, *Gfp* and phospholamban expression in wild type (WT) (n = 6) and *Lmna*^{H222P/H222P} mice (n=6), normalized to *Gapdh* (C) Micrographs showing α -actinin and GFP labeling in hearts from 24 weeks old *Lmna*^{H222P/H222P} treated with an AAV9-null (*Lmna*^{H222P/H222P} null) and *Lmna*^{H222P/H222P} treated with an AAV9-sh*Sln*-GFP (*Lmna*^{H222P/H222P} sh*Sln*). Nuclei are counter-stained with 4',6-diamidino-2-phenylindole (dapi). (D) **Left ventricular progression after AAV9 treatments.** Fractional shortening evolution (LVFS %), end-diastole dimension (LVEDD), end-systole dimension (LVESD) from 12 to 24 weeks in male wild type (WT) (n=3), *Lmna*^{H222P/H222P} treated with an AAV9-null (*Lmna*^{H222P/H222P} null) (n=5) and *Lmna*^{H222P/H222P} treated with an AAV9-sh*Sln*-GFP (*Lmna*^{H222P/H222P} sh*Sln*) (n=4). Data are represented as median [Q1; Q3] *p \leq 0.05 : p-value obtained using a Wilcoxon test.

Morales Rodriguez et al. Supplemental Figure 1



Supplemental Figure 1. Left ventricular fractional shortening progression | Fractional shortening evolution (LVFS %) from 12 to 24 weeks in male *Lmna*^{H222P/H222P} (n=6) and wild type (WT) (n=6) mice. Data are represented as median [Q1; Q3] **p<0.01 : p-value obtained using a Wilcoxon test.



Discussion

Chapter 3: Discussion, conclusions and recommendations

The investigation in this thesis aimed to dissect the pathomechanisms implicated in *LMNA* cardiomyopathy. A better understanding of these mechanisms is of paramount to identify new therapeutic targets for future treatments.

The present work brings new insights in the role played by oxidative stress in the progression of *LMNA* cardiomyopathy and highlights a novel approach to ameliorate the left ventricular function. The second part of this work sheds light on the importance of calcium regulation and the role played by sarcolipin in the pathophysiological mechanisms driving the impaired cardiac function in *LMNA* cardiomyopathy.

1. Oxidative stress and lamins: new therapeutic avenues?

Due to the pathological effect of oxidative stress in different laminopathies (Caron et al., 2007b; Malhas and Vaux, 2011; Barascu et al., 2012; Lattanzi et al., 2012) as well as in cardiomyopathies (Dhalla et al., 2000; Maack et al., 2003; Sawyer, 2011; Münzel et al., 2017), it was hypothesized that increased oxidative stress could participate in the progression of *LMNA* cardiomyopathy and thus its reduction could have beneficial effects.

The main findings of the first part of this investigation demonstrate that the redox imbalance is implicated in the worsening of the cardiac function. It was observed a decreased in glutathione content and in the expression of components of the antioxidant system - such as glutathione peroxidase 1 (GPX1)- in parallel to an increase in the expression of NADPH oxidase (NOX) and carbonylation profiles were observed in the *Lmna*^{H222P/H222P} mouse model hearts. These findings are similar to the ones observed in other laminopathies

such as partial lipodystrophies (Caron et al., 2007b; Verstraeten et al., 2009), amyotrophic quadricipital syndrome with cardiac involvement (Charniot et al., 2007), EDMD (Pekovic et al., 2011; Niebroj-Dobosz et al., 2017), HGPS (Viteri et al., 2010) and restrictive dermopathy (Viteri et al., 2010). It is then clear that defective lamins induce an increased ROS production, but the link between altered lamins and oxidative stress remains to be fully elucidated. Notwithstanding the different hypothesis, not mutually exclusive, have been proposed to explain the implication of lamins in the redox imbalance (this implication is further discussed in **section 3**).

There are several potential explanations for the implications of excessive oxidative stress in the progression of *LMNA* cardiomyopathy. ROS have been described as important mediators of cellular functions (Schieber and Chandel, 2014), such as proliferation, inflammation, senescence and protein degradation via the regulation of different proteins or protein complexes: p53, pRB, Sirtuine 1, mTORC or NF κ B, all of them being altered in different cardiomyopathies (Münzel et al., 2017). With regards to lamins, ROS regulate their expression, polymerization and stability (Shimi and Goldman, 2014; Torvaldson et al., 2015). The increased oxidative stress alters all these functions, mainly by altering the post-translational modifications summarized in **Figure 8** (Kosako et al., 2009; Ragnauth et al., 2010; Vos et al., 2011; Pekovic et al., 2011; Torvaldson et al., 2015). However, not only lamin functions can be altered by acute oxidative stress but also cardiac function. Physiological oxidative stress in the heart is implicated in cardiomyocytes differentiation and proliferation, excitation contraction coupling, regulation of blood flow and adaptation to exercise. Furthermore, pathological oxidative stress leads to reduced cardiac contractility, arrhythmias and cell death via different mechanisms summarized in **Figure 9** (Burgoyne et al., 2012; Rababa'h et al., 2018).

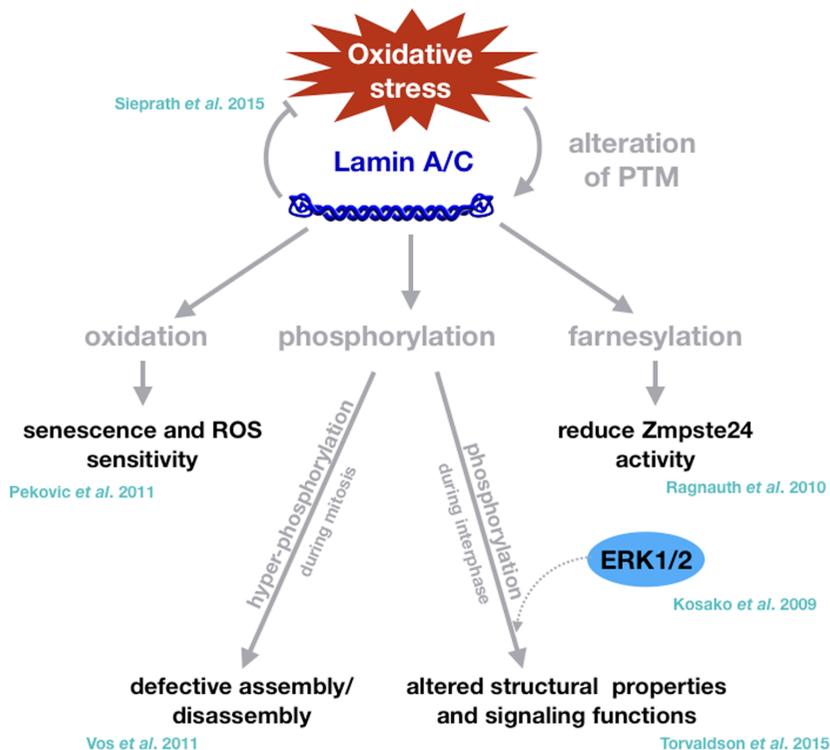


Figure 8. Schematic representation of post-translational modifications of lamins controlled by oxidative stress. **Oxidation:** different residues in the C-terminal tail of lamin-A have an essential role in cellular fitness. Their excessive oxidation alters lamin sensitivity to ROS and is involved in cellular senescence. **Phosphorylation:** lamins are hyper-phosphorylated during mitosis and phosphorylated during interphase assuring their functions. Excessive oxidative stress or lamin mutations alter the phosphorylation leading to defective assembly/disassembly mechanisms. **Farnesylation:** lamin farnesylation is essential for maturation. Excessive oxidative stress can alter this maturation process by reducing Zmpste24 activity.

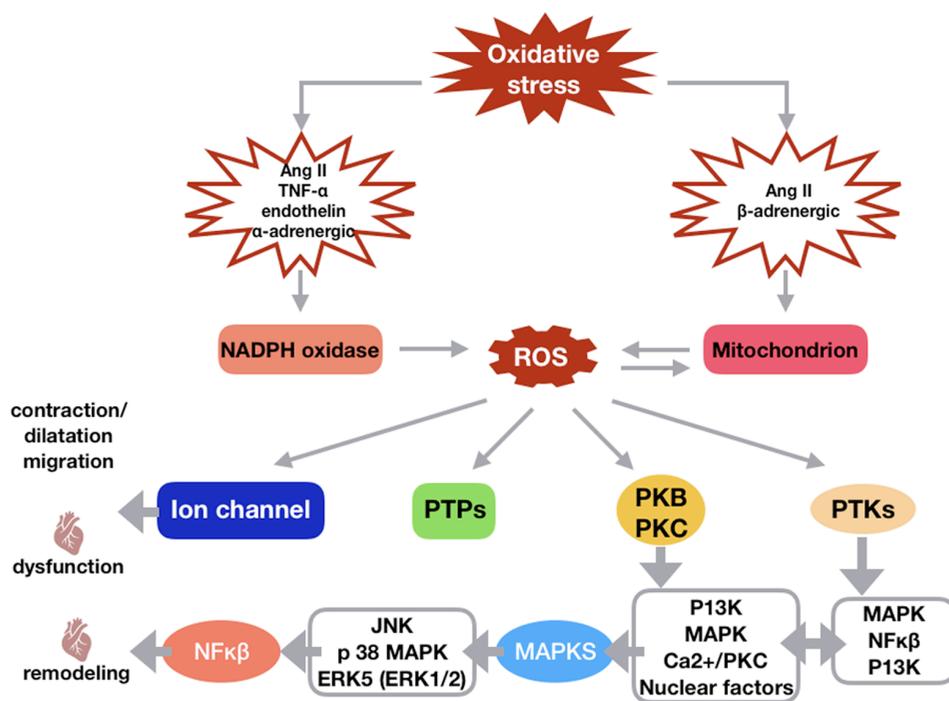


Figure 9. Schematic representation of cardiac oxidative stress mediated modulations. Oxidative stress induces the over production of ROS via NADPH oxidases or mitochondrion activity. The produced ROS regulate the activity of different proteins as: ion channels, protein tyrosine phosphatases (PTPs), protein kinases B (PKB) and C (PKC) and protein tyrosine kinases (PTKs).

Regardless of the initial causal agent (*LMNA* mutations cause excessive oxidative stress, or excessive oxidative stress induces lamin defects), the excessive oxidative stress should be treated to palliate its pathologic effects. Even if glutathione is critical to fight oxidative stress, it is an extremely limited resource due to its scarce bioavailability. The administration of NAC – glutathione’s precursor- was therefore proposed as a therapeutic option, (Schmitt et al., 2015). The current study demonstrates that a one-month-long N-acetyl cysteine (NAC) treatment was beneficial against cardiac dysfunction progression in *Lmna*^{H222P/H222P} mice. This is in line with the use of NAC treatments in other cardiomyopathies, such as hypertrophic cardiomyopathy (HCM) (Marian et al., 2006; Reyes et al., 2017), heart failure (Giam et al.,

2016), post myocardial infarction (Adamy et al., 2007) and vascular diseases derivate from diabetes (Dludla et al., 2018). Several parameters implicated in the cardiac pathology such as the amount of carbonylated proteins or the fibrosis were also reduced with NAC treatment, which could be explained in part by the restoration of physiological glutathione levels. Myocardial fibrosis in patients with similar muscular dystrophies is as a significant contributor to the development of cardiac function defects, which remains the main cause of death for most of them (Frankel and Rosser, 1976; Verhaert et al., 2011). The current study highlighted the beneficial effect of NAC treatment towards a significant fibrosis reduction. So, ameliorating this parameter is crucial to present NAC as a potential therapeutic strategy. These beneficial effects of NAC treatment are in accordance with the ones reported in other models (Marian et al., 2006; Giam et al., 2016). Hence, due to the pharmacokinetics and the absence of toxicity, NAC should be taken in consideration for the treatment of *LMNA* cardiomyopathy.

Thus, in order to translocate the best therapeutic strategy to patients, some aspects of this treatment required further discussion/investigation. This study has defined NAC as a protecting agent against cardiomyopathy progression, however it does not present a 100% curative effect. In fact, even after the restoration of physiologic glutathione levels, no fractional shortening restoration was observed in the treated *Lmna*^{H222P/H222P} mice. It can be hypothesized that NAC treatment prior to the onset of the cardiac dysfunction could protect the heart from degeneration. However, when NAC was given after LV dilatation and decreased cardiac ejection fraction, it is not beneficial. The results from a clinical trial on cardiac injury after abdominal aortic aneurysm repair support this hypothesis. Where the administration of NAC prior to surgery protected the patients from oxidative stress (Mahmoud and Ammar, 2011). It would be of great interest to define if treating these mice at a pre-symptomatic stage inhibits or delays the outbreak of the pathology.

Another interesting point would be to assess the redox state of skeletal muscle before and after the treatment, in order to determine whether the beneficial effects are organ-specific or not. If there is an amelioration of skeletal muscle redox state, this treatment would become even more interesting for the treatment of *LMNA* cardiomyopathy but also for a broader range of muscular dystrophies with or without cardiac impairment.

Nevertheless, since NAC is not specific to muscular tissue, other therapies should also be taken into consideration. Nowadays most attempts to reduce oxidative stress have been futile. This can be explained by an insufficient reduction in the required cellular type or by subcellular compartment or it might be due to a non-selective ROS modulation. ROS are essential for different signaling pathways, so a non-selective modulation could interfere with physiological ROS-dependent signaling pathways (Dao et al., 2015). However a large number of innovative options should be taken in consideration rather than classical antioxidant supplementations. As an example, it would be interesting to identify the proteins responsible for the carbonylation to define targeted therapies. Similar approaches modulating the expression of antioxidant systems led to encouraging results in amyotrophic lateral sclerosis (Nanou et al., 2013). Other possibility would be to specifically target NOX2 and NOX4 as previously described in other systems (Stielow et al., 2006; Jucaite et al., 2015).

In summary, the results obtained in of *Lmna*^{H222P/H222P} mouse presented in this first part showed that increased oxidative stress is implicated in the cardiac phenotype progression of *LMNA* cardiomyopathy and endorse NAC as a possible therapeutic option for patients.

2. Sarcolipin: key regulator of pathological Ca²⁺ homeostasis in LMNA cardiomyopathy?

Given the importance of intracellular calcium regulation in cardiac function and the implication of calcium imbalance in cardiomyopathies (Kranias and Bers, 2007), the second part of this thesis focused in the regulation of calcium homeostasis in *LMNA* cardiomyopathy. Pulling this thread, the expression levels of different calcium regulators were analyzed from the results of a previously performed microarray DNA analysis on the *Lmna*^{H222P/H222P} mice (Muchir et al., 2007b). Interestingly, *Sln* was among the ten most affected genes, encoding for sarcolipin, a main regulator of the SERCA pump. The early over-expression of *Sln* give rise to the hypothesis which considers that Ca²⁺ homeostasis could be altered and *Sln* could be the driving force of this imbalance.

The main findings from the second part of this investigation demonstrate that sarcolipin was up regulated along with the progression of cardiomyopathy in hearts from *Lmna*^{H222P/H222P} mice, leading to a prolonged Ca²⁺ recapture time due to an alteration of SERCA2a and/or NCX1 function. SERCA's impaired function was described in cardiac sarcolipin over-expression models (Asahi et al., 2004; Babu et al., 2005, 2006), confirming the implication of sarcolipin in delayed calcium recapture. In accordance to these studies and despite of the reduction of Serca2a activity, its expression was not altered. This confirms the fact that the impaired Ca²⁺ recapture was not due to a defective expression of SERCA2a but rather of modulation of its activity by sarcolipin. Though an altered expression of other calcium regulators such as NCX1 or phospholamban was observed in hearts from *Lmna*^{H222P/H222P} mice, modifications have never been reported so far. These alterations were observed both *in vivo*, in the ventricles of *Lmna*^{H222P/H222P} mice, and *in vitro* in the sarcolipin-over expression cardiac cellular models (H9C2 and HL-1

expressing FLAG epitope-tagged sarcolipin). Concerning NCX1 overexpression, it is complicated to determine if this is common to all sarcolipin over-expression models because previous studies have not reported its expression levels. It can be speculated that NCX1 expression can be increased to attenuate the intracellular calcium overload, as reported in other cardiomyopathies (Wang et al., 2009; Lu et al., 2011).

Regarding the phospholamban reduced expression, not all the studies reported the same results. A theory of a compensatory mechanism was proposed by Gramolini *et al*, suggesting that to compensate sarcolipin over-expression, mechanisms leading to a regulation of sarcolipin or phospholamban activities could occur (Gramolini et al., 2006). Most of the studies with sarcolipin alterations reveal an unaltered phospholamban expression level not only in cardiac muscle (atrial or ventricular) but also in skeletal muscle (Asahi et al., 2004; Babu et al., 2005, 2006, 2007a; Ottenheijm et al., 2008; Xie et al., 2012; Shanmugam et al., 2015; Voit et al., 2017), rejecting the hypothesis of a compensatory regulation. Conversely, different studies lean towards a compensatory regulation between phospholamban and sarcolipin expression (Babu et al., 2007b; Shanmugam et al., 2015). This hypothesis is more in accordance with the results obtain during my thesis.

However a third possibility could globally explain the results (summarized in **figure 10**). It can be speculated that the compensatory mechanisms exist but in a two steps dependent mechanism. In the first step the regulation of sarcolipin/phospholamban activity depends on the phosphorylation control. If sarcolipin is overexpressed, an increased phosphorylated phospholamban would be observed. This can be endorsed by the increased phospho-phospholamban observed in the hearts of sarcolipin over-expression mouse model (Shanmugam et al., 2015), as well as by the results obtained during my thesis. Then, if the activation/inactivation of phospholamban is not enough to re-establish normal SERCA2a function, the

expression of sarcolipin or phospholamban could be altered. If these two steps regulation takes place, it is then essential to know how sarcolipin can be activated/deactivated. Due to the structural and functional similarities of sarcolipin and phospholamban, different studies have analyzed the post-translational modifications of sarcolipin. Recent studies have shown that phosphorylation of threonine 5 (conserved residue among mammals) could be in charge of its function (Bhupathy et al., 2009; Shanmugam et al., 2015).

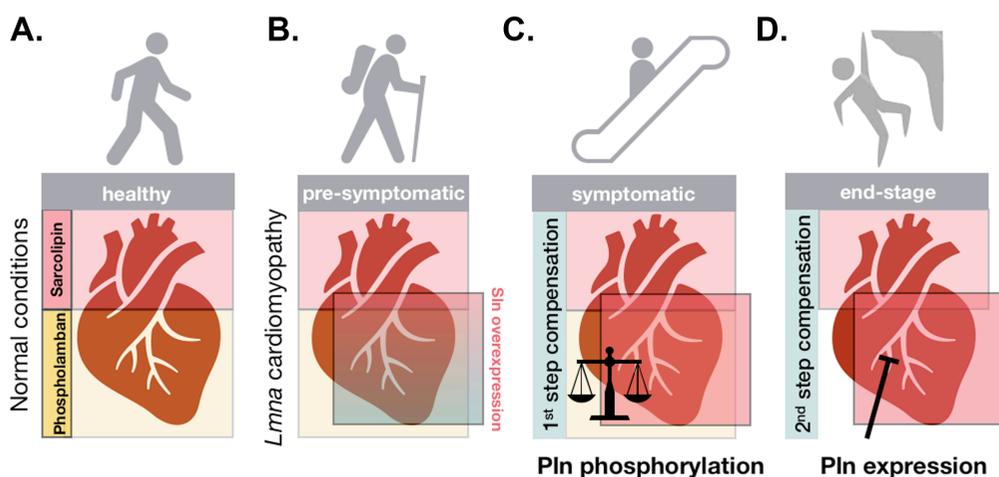


Figure 10. Schematic representation of the hypothesis of the 2-step-regulation compensation mechanism. **A.** Normal condition: sarcolipin is expressed on atria and phospholamban in ventricles. **B.** *Lmna* cardiomyopathy: phospholamban is expressed on ventricles whether sarcolipin is expressed in both atria and ventricles. **C.** First step of the compensatory mechanism: regulation of phospholamban by phosphorylation. **D.** Second step of the compensatory mechanism: inhibition of phospholamban expression.

All the results mentioned above have led us to the following investigation: AAV-mediated down-regulation of sarcolipin could lead to a reduced cardiac progression of *LMNA* pathology.

Contrarily to what was hypothesized, the treatment with shRNA-*Sln* did not mitigate the cardiac progression in *Lmna*^{H222P/H222P} mice. However, the expected beneficial effect of this AAV-mediated therapy was observed in

recent studies performed on *mdx* mouse (Voit et al., 2017). Taking into account the available evidence, it can be proposed that a possible reason that an improvement in cardiac function was not detected in our current study, due to the low transduction level (20%). The transduction efficiency was enough to reduce sarcolipin over-expression up to 40%, but it may not be sufficient to ameliorate the cardiac phenotype. Increasing the efficiency of the treatment could lead to the expected beneficial effect. Additionally the sequence targeted in this study is different to the one targeted by Voit *et al.* (Voit et al., 2017), and that fact could also explain the different results. It would be of great interest to target the same sequence as Voit *et al.* to determine the implication of sarcolipin in *LMNA* cardiomyopathy.

Sarcolipin alterations have been observed in a large panel of myopathies such as: amyotrophic lateral sclerosis (Calvo et al., 2012), distal myopathy (Ottenheijm et al., 2008), Duchene muscular dystrophy (Schneider et al., 2013), myotonic dystrophy (Osborne et al., 2009), centro-nuclear myopathy (Nakagawa et al., 2005; Fajardo et al., 2017), dynamin-2 dependent centro-nuclear myopathy (Liu et al., 2011), dysferlinopathies (Campanaro et al., 2002) and so, in corticosteroide-induced myopathies (Gayan-Ramirez et al., 2000). Thus, a further possibility to explain the absence of cardiac improvement due to sarcolipin regulation could be that sarcolipin is not directly linked to the *LMNA* pathology progression but it is rather a marker of a defective striated muscle.

In summary, the data presented in the second part of our investigation confirm that sarcolipin overexpression is sufficient to create a calcium homeostasis imbalance. Additionally it suggests a novel model for A-type lamins in the regulation of calcium homeostasis. Further investigation is required to determine the exact role of sarcolipin in the progression of *LMNA* cardiomyopathy and to determine if sarcolipin could be a novel and universal muscle therapeutic target.

3. Cross- link between defective lamins, increased oxidative stress and calcium imbalance.

This original work has shown that defective regulation of oxidative stress and altered calcium homeostasis are implicated in the progression of *LMNA* cardiomyopathy. It is now clear that the reduction of oxidative stress is a potential therapeutic option for the disease. On the other hand, the implication of sarcolipin in the pathomechanisms requires further investigation. However it has become clear that sarcolipin plays a critical role in the impaired cardiac calcium recapture and the altered expression of different calcium regulators.

It is currently known that there is a close regulation between lamins functions, production of ROS and calcium homeostasis. Moreover it is clear that these close interactions can be altered in pathological situations, inducing a pathological regulation loop of the other components.

The different roles and effects induced by alterations in one of the components of the system are summarized in **figure 11**. The possible interplays between these three systems in the case of laminopathies would be discussed in the next sections.

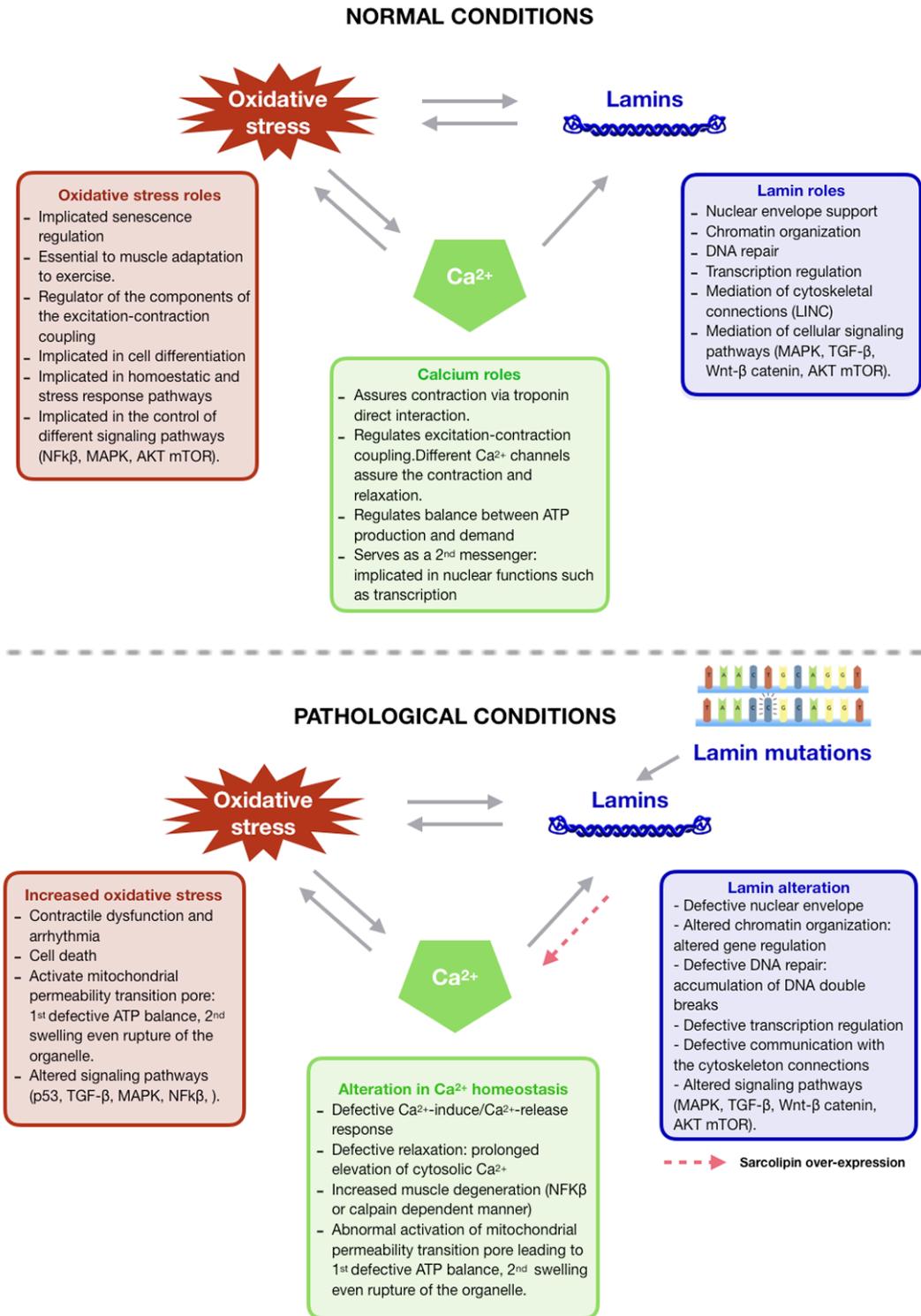


Figure 11. Schematic summary of the different interactions between lamins, calcium homeostasis and oxidative stress in normal and pathological conditions.

3.1 Dysfunctional lamin lead to oxidative stress

Although it is clear that *LMNA* mutations lead to increased oxidative stress, the exact mechanism of it remains unclear. Different hypotheses not mutually exclusive have been proposed to explain this interplay. 1) Vos *et al* proffered showed that intermittent ruptures of the nuclear envelope (due to A-type lamin mutations) lead to loss of cellular compartmentalization inducing a mis-localization of transcription factors altering the regulation of different genes, among which Oct-1 (Vos *et al.*, 2011) defined as one of the main actors of lamin-induced oxidative stress (Malhas *et al.*, 2009). The loss of cellular compartmentalization could also explain the localization of mitochondria inside of the nucleus, observed in some laminopathies patients, increasing by rapprochement the risk of oxidative damage (Sylvius *et al.*, 2005; Vos *et al.*, 2011). 2) A second hypothesis was proposed, based on defective lamin-chromatin interaction due to *LMNA* mutations inducing ATR mislocalization (Manju *et al.*, 2006). The ATR mislocalization could be responsible of the DNA double breaks accumulation, that has been described as implicated in ROS production (Kang *et al.*, 2012). 3) A third hypothesis, based on the presence of a nuclear shield of antioxidant enzymes (Fabrini *et al.*, 2010) anchored to the nucleus by the LINC complex, propose that *LMNA* mutations alter A-type lamins scaffolding role of the LINC-complex inducing nesprins mislocalization leading to a local decline of antioxidants around the nucleus, which could in turn elicit a higher susceptibility to oxidative stress. 4) A fourth hypothesis was also proposed focusing on the role of lamins as signaling pathways regulators. Lamins play an important role in the regulation of signaling pathways mediated by SIRT1 (Liu *et al.*, 2012), NF- κ B (Osorio *et al.*, 2012) and mTORC1 (Ramos *et al.*, 2012). These pathways are known to be involved in regulating oxygen metabolism and oxidative stress. Though in many cases, direct biochemical evidence has yet to be provided, however

these studies imply that A-type lamins may regulate a cellular response to oxidative stress through these pathways.

Even if the A-type lamin mutations are initially responsible for the increased oxidative stress, there is a positive retro-control loop inducing ROS production that plays an important role in the observed phenotypes. The recapitulations of the different hypothesis of the contribution of lamin mutations to the oxidative stress are summarized in **figure 12**.

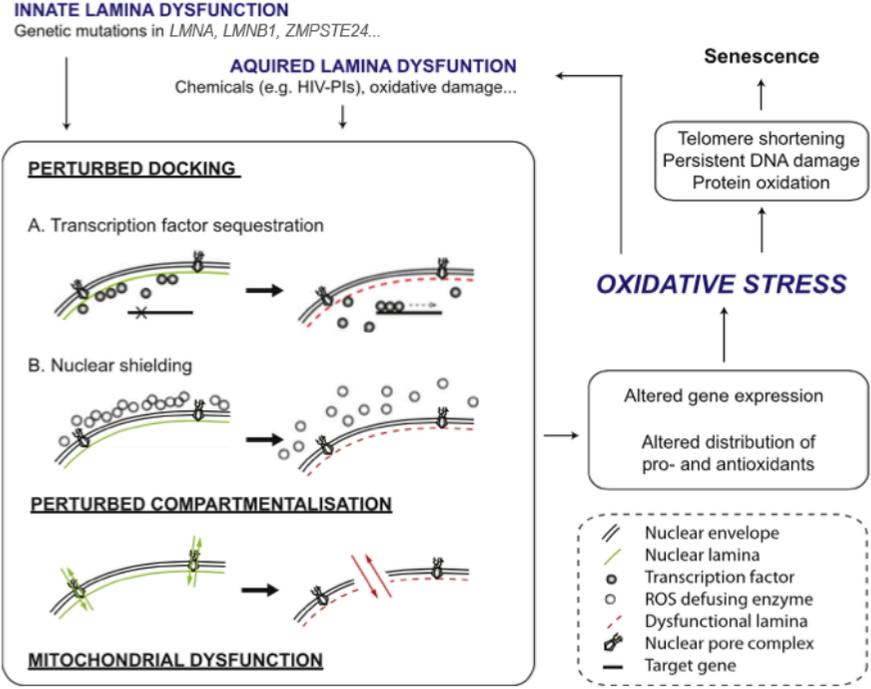


Figure 12. Recapitulation of different hypothesis Figure extracted from: Sieprath *et al.* 2012 – “*Lamins as mediators of oxidative stress*”. Original legend: Framework for reciprocal interactions between lamins and oxidative stress. Genetic (innate) or non-genetic (acquired) mechanisms cause lamina dysfunction, which leads to an altered affinity (docking) for redox-responsive transcription factors and/or ROS defusing enzymes, reduced compartmentalization potential (ruptures) or mitochondrial dysfunction. This causes changes in stress-responsive gene expression and/or spatial redistribution of pro- (mitochondria, NOX) and anti-oxidants (CAT, SOD. . .), inducing a state of oxidative stress. Chronic oxidative stress induces telomere shortening, protein oxidation and persistent DNA damage, eventually heralding cellular senescence.

3.2 Dysfunctional lamins lead to an altered calcium homeostasis

The link between lamins and altered calcium homeostasis is harder to be proved. The present study can suggest that a mutation of lamin A/C would be directly responsible of a sarcolipin over-expression implicated in the altered calcium homeostasis. However this theory requires further investigation.

Besides this hypothetical role, the implication of calcium as a secondary messenger regulating a wide range of cellular activities has been described long ago (Rasmussen et al., 1976). The effect of the accumulation of this secondary messenger in the nucleus has also prompted great interest, and its roles in the nucleus have been thoroughly reviewed (Gomes et al., 2006).

Echevarría *et al.* proposed the existence of a nucleoplasmic reticulum, that has been confirmed by other groups (Echevarría et al., 2003; Malhas et al., 2011). It can be speculated that lamins are implicated in the regulation of these nucleoplasmic reticulum. Then, a disruption in the nuclear envelope would lead to increased calcium in the nucleus and defective signalling pathways. This hypothesis can be sustained by the results obtained by microscopy analysis showing that lamin is implicated in the formation of nucleoplasmic reticulum (Drozd et al., 2017). Therefore a defect in lamins would trigger a domino effect that would induce an alteration of the nuclear calcium and subsequently an alteration of the cytoplasmic calcium.

In addition to all the possibilities mentioned above, the role of lamins as a signalling regulator cannot be neglected. A-type lamins are implicated in the regulation of different pathways that could be responsible of the altered calcium homeostasis. This option appeal to be the most realistic to explain the implication in lamins in calcium imbalance.

3.2 Increased oxidative stress lead to dysfunctional lamins and calcium imbalance

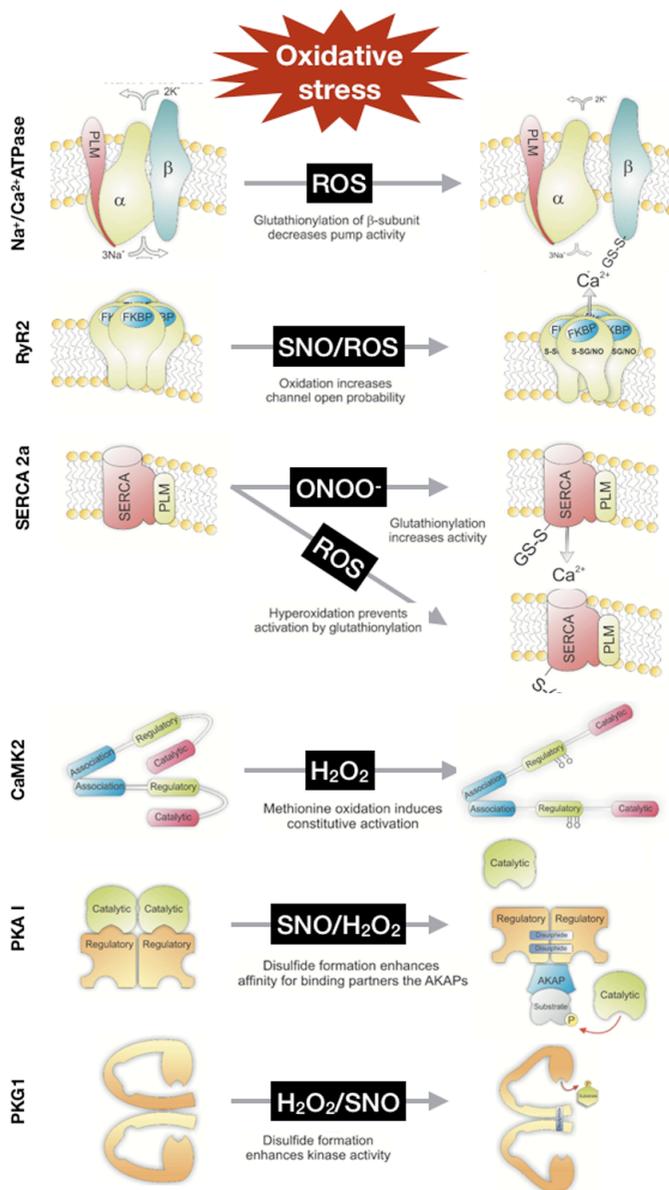
The implication of oxidative stress in the regulation of lamins is recapitulated in **figures 8 and 12**. As mentioned before the increase oxidative stress is a mayor actor of the physiopathology of laminopathies. Redox signaling is implicated in the development of many pathological components of the heart phenotype, such as contractile dysfunction, calcium dysregulation, cardiomyocyte hypertrophy, cell death, arrhythmia, fibrosis, and chamber dilation (Burgoyne et al., 2012). It is interesting to note that important amounts of ROS can trigger the activation of different signaling cascades among which are MAPKs, leading to induced dilated cardiomyopathies (Cardinale et al., 2006; Zeglinski et al., 2011). For *LMNA* cardiomyopathy it can be speculated that the dual regulating role of oxidative stress (regulation of lamins functions and regulation of calcium balance) is in charge of the progression of the pathology. Thus, previous studies, revealed that MAPKs pathway was one of the most altered in *Lmna*^{H222P/H222P} mouse (Muchir et al., 2007b, 2012) so it can be speculated that the increased oxidative stress can be in part responsible of this signaling pathway alteration.

As far as oxidative stress implication for the direct regulation of calcium balance is concerned, it has been shown that elevated ROS levels can alter calcium homeostasis through plasma membrane proteins, mitochondria and intracellular calcium channels (Duchen et al., 2008). The redox signaling can target different calcium regulators summarized in **table 5** (cf. introduction section). A summary of all the possible alterations of the different calcium regulators is presented in **figure 13**.

Due to the results obtain during my thesis, we can speculate that the altered functions of SERCA2a and NCX1 in *Lmna*^{H222P/H222P} mouse can be in part explained by an increase oxidative stress. Since SERCA2a activity can be regulated by oxidative stress (high concentrations of oxidative stress induces

an inhibition), it will be interesting to determine the SERCA2a activity after the NAC treatment.

Thus, the activity of the RyR channel has not been investigated, however it would be of great interest to further investigate its possible contribution to the pathology.



Oxidation of the Na⁺/K⁺ ATPase β1 subunit decreases pump activity, which can be reversed by β3 adrenoceptor stimulation.

Ryanodine receptor (RyR) 2 oxidation to the S-nitrosylated or glutathiolated state enhances channel open probability.

Glutathiolation of Cys674 on SR Ca-ATPase (SERCA) augments its activity but is prevented by the hyperoxidation of this residue under pathological conditions. ROS indicates reactive oxygen species; ONOO, peroxynitrite; PLM, phospholamban; SNO, S-nitrosothiol.

Oxidation of methionine residues in the Ca/calmodulin-dependent kinase II (CaMKII) regulatory domain prevents reassociation of the autoinhibitory complex and generates increased catalytic activity independent of Ca/CaM.

Oxidation of the type 1 regulatory subunit dimer of protein kinase A (PKA) forms 2 intermolecular disulfides that enhance kinase binding affinity for A-kinase anchoring proteins (AKAPs), bringing it into close proximity with its substrates.

Protein kinase G (PKG) 1α oxidation leads to intermolecular disulfides de formation that increases kinase affinity for substrate, leading to translocation to the membrane and myofilaments, as well as augmented target phosphorylation. SNO indicates S-nitrosothiol.

Figure 13. Modulation of excitation contraction coupling proteins and its regulators
Figure adapted from: Burgoyne *et al.* 2012 – “Redox Signaling in Cardiac Physiology and Pathology”.

3.4 Altered calcium homeostasis alters lamin functions and induces oxidative stress

Calcium has been described as a main contributor to cardiomyopathies and muscular dystrophies due to its key role in muscle contraction as its function as a second messenger (Kranias and Bers, 2007; Burr and Molkentin, 2015). With respect to lamins, calcium has been shown to promote farnesylation, essential for the maturation of lamins (Kalinowski et al., 2013). No further direct relationships between calcium and lamins have been described.

Per contra its involvement of calcium in response to oxidative stress has been extensively investigated (Ermak and Davies, 2002; Görlach et al., 2015). The increase in cytoplasmic calcium is known to regulate ROS primarily through disrupting mitochondrial function in a phospholipase dependent manner (Nethery et al., 2000; Gong et al., 2006). In physiological conditions, calcium regulates balance between ATP production and ATP-demand. However, in pathological conditions, the intracellular calcium overload induces mitochondrial permeability transition pores. This phenomenon is responsible for an increased production of ROS, which aggravates the opening of the non-specific pores in the mitochondria, finally leading to a mitochondrial dysfunction or destruction responsible of cellular death.

All together, we can conclude that it is impossible to blame calcium or oxidative stress as the main cause of the disease since both are self-regulating. We can therefore say that oxidative stress and the alteration of calcium contribute to the progression of the pathology and should be finely regulated to prevent the onset of the symptoms.

Further studies are required to determine if the restoration of normal oxidative stress levels by NAC treatment (or other treatments) have an effect in the regulation of calcium homeostasis. In the same line, studying the redox

state after controlling calcium homeostasis by restoring normal sarcolipin levels (or by other methods) would be of great interest.

Nevertheless we will still be missing the direct link between *LMNA* mutations and the excessive oxidative stress, as well as the direct link between *LMNA* mutations and the over-expression of sarcolipin leading to an altered calcium homeostasis. It is now a challenge to the field to determine those links.

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