School of Biology and Molecular Medicine

PhD Course in Human Biology and Medical Genetics

XXIX° cycle

Coordinator: Prof. Antonio Pizzuti

Next generation sequencing for the molecular analysis of sarcomeric and non-sarcomeric genes in patients with hypertrophic cardiomyopathy

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To my parents

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ABSTRACT

Hypertrophic cardiomyopathy (HCM) is a common genetic heart disorder characterized by unexplained left ventricle hypertrophy associated with non-dilated ventricular chambers. Several genes encoding heart sarcomeric proteins have been associated to HCM, but a small proportion of HCM patients harbor alterations in other non-sarcomeric loci. The variable expression of HCM seems influenced by genetic modifier factors and new sequencing technologies are redefining the understanding of genotype-phenotype relationships, even if the interpretations of the numerous identified variants pose several challenges. We investigated 62 sarcomeric and non-sarcomeric genes in 41 HCM cases and in 3 HCM-related disorders patients. We found that 82% of the patients harbored at least one rare nsSNV: 11% of the patients showed only sarcomere nsSNVs, 20% of cases harbored at least one sarcomeric nsSNV with at least a desmosomal one and 14% displayed at least one desmosomal nsSNV but no other sarcomere change. We reported an association between desmosomal variations and the pathogenesis of HCM that has not been described to date. We employed an integrated approach that combines multiple tools for the prediction, annotation and visualization of functional variants. Several different methods were employed to predict the functional consequences of alleles that result in amino acid substitutions, to study the effect of some variants over the splicing process and to investigate the impact of these changes respect to the evolutionary conservation. Genotype-phenotype correlations were carried out for inspecting the involvement of each gene in age onset and clinical variability of HCM. Statistical analyses revealed an inverse correlation between the number of nsSNVs and age at onset, and a relationship between the clinical variability and number and type of variants. Then, we describe the clinical, pathological, and molecular features of the novel *LAMP2* c.453delT mutation in one of our HCM-related disorders patients affected by Danon disease characterized by severe hypertrophic cardiomyopathy, mild intellectual impairment and rapid progression to heart failure, requiring heart transplant. Immunohistochemical analysis of LAMP2 in the explanted heart revealed a mosaic pattern of distribution, with discrete clusters of either stained or unstained cardiac myocytes, the latter being more frequent in the septum. Interestingly, multiple foci of microscarring were found on histology in the Left Ventricle (LV) free wall and septum. Our findings suggest that several features may contribute to the early and severe cardiac phenotype in female patients affected by Danon disease. In conclusion, this work aims to extend the mutational spectrum of HCM and to contribute in defining the molecular pathogenesis and inheritance pattern(s) of this

condition. Besides, we delineate a specific procedure for the identification of the most likely pathogenetic variants for a next generation sequencing approach embodied in a clinical context.

1. INTRODUCTION

1.1 THE HEART

The heart is a central organ in the circulatory system and it is indispensable for normal organism homeostatis by providing a constant supply of blood to tissues, wich carries oxigen and nutrients and removes carbon dioxide and waste products (Cardiac Tissue Engineering: Principles, Materials, and Applications; Ruvinov, Sapir, Cohen, 2012). The human heart is situated in the protective thorax, further back to the sternum and costal cartilages, and rests on the superior surface of the diaphragm. The heart assumes an oblique position in the thorax, with two-thirds to the left of midline. The heart and great vessels are covered by the sternum and central part of the thoracic cage. The apex of the heart usually hidden in the 5th intercostal space, just medial of the midclavicular line. All four heart valves stay well protected behind the sternum. The sounds of the individual valves closing are best heard at ausculatory sites to which their sounds are transmitted. The bicuspid (mitral) valve is heard at the apex of the heart in the region of the 4th or 5th intercostal spaces on the left near the midclavicular line. The tricuspid valve can be heard along the left margin of the sternum at the level of the 4th or 5th intercostal space. The pulmonary valve is situated along the left border of the sternum in the 2nd intercostal space. The aortic valve is fixed at the 2nd intercostal space on the right sternal border (Handbook of Cardiac Anatomy, Physiology, and Devices, Second Edition, Paul A. Iaizzo Editor, 2009).

The heart pumps blood through blood vessels and to realize that, the heart beats about 100.000 times every day, pumping 5 liters of blood each minute. Due to the central function of the heart in sustaining life and normal homeostatis of the body, the insufficient ability of the heart to self-rigenerate after damage results in a progressive deterioration in the heart function.

During this process, the heart undergoes to some structural and biochemical changes, leading to reduced contractility and heart failure (Cardiac Tissue Engineering: Principles, Materials, and Applications; Ruvinov, Sapir, Cohen, 2012).

1.2 CARDIAC MUSCLE STRUCTURE

The internal anatomy of the heart is composed by four chambers (Fig.1). The two upper receiving chambers are the atria and the two lower pumping chambers are the ventricles. The

role of the ventricle is to pump oxygenated blood into the systemic circulation to other tissues of the body except the alveoli of the lungs. The role of the right ventricle is to pump deoxygenated blood into the pulmonary circulation to the alveoli of the lungs.

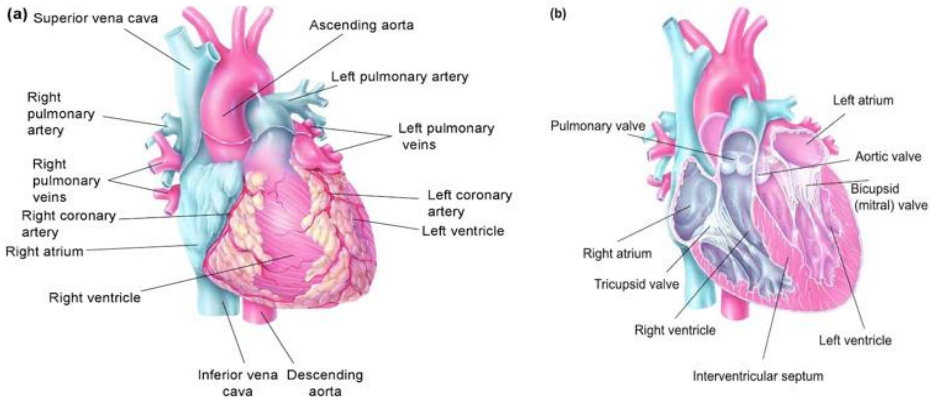


Figure 1. Heart structures. **A**. Anterior external view of the heart. **B.** Major internal features of the heart (Cardiac Tissue Engineering: Principles, Materials, and Applications; Ruvinov, Sapir, Cohen, 2012)

There is one-way flow of blood through the heart; this flow is maintained by a set of four valves. The atrioventricular or AV valves (tricuspid and bicuspid) allow blood to flow only from atria to ventricles; the semilunar valves (pulmonary and aortic) allow blood to flow only from the ventricles out of the heart and through the great arteries (Fig.1) (Handbook of Cardiac Anatomy, Physiology, and Devices, Second Edition, Paul A. Iaizzo Editor, 2009).

The wall of the heart consists of three layers: the epicardium (external), the myocardium (in the middle) and the endocardium (the inner layer).

The epicardium appears transparent and it is constitutes by mesothelium and connective tissue that allows a smooth, slippery texture to the outermost surface of the heart. Myocardium is the cardiac muscle tissue and constitutes about 95% of the heart mass and is responsible for its pumping action. The innermost endocardium is a thin layer of endothelium overlying a thin layer of connective tissue. It provides a smooth lining for the chambers and covers the valvesof the heart (Cardiac Tissue Engineering: Principles, Materials, and Applications; Ruvinov, Sapir, Cohen, 2012).

1.3 MYOCARDIUM AND THE MYOFIBRILLAR STRUCTURE

The human myocardium is composed approximately by 2.5 billion cardiomyocytes (the muscle cells of the heart), it is possible to find these cells only in the heart and they are

different from the skeletal and smooth muscle cells (Cardiac Tissue Engineering: Principles, Materials, and Applications; Ruvinov, Sapir, Cohen, 2012).

Cardiomyocytes are shorter than skeletal myocytes and have one centrally located nucleus, sometimes they have fewer nuclei (Braunwald *et al.;* 1971). The cardiac muscle fibers are shorter than skeletal muscle fibers in lenght and less circular in the transverse section. A tipical cardiac muscle fiber is 50-100 µm long and has a diameter of about 14 µm. Each muscle fiber connects to the sarcolemma (plasma membrane) with characteristic tubules (T-tubule). At these T-tubules, the sarcolemma is connected with a large number of calcium channels wich permit calcium ion exchange. The flux of calcium ions into the muscle cells stimulates an action potential, wich allows the cells to contract. Electrical excitation of cardio cells leads to contraction of the heart through the process of excitation-contraction coupling (ECC). The second messenger, Ca^{2+}, is essential for cardiac electrical activity and is the principal activator of the myofilaments (Cardiac Tissue Engineering: Principles, Materials, and Applications; Ruvinov, Sapir, Cohen, 2012).

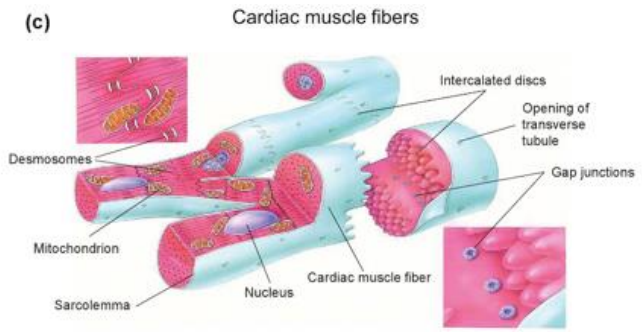


Figure 2. Cardiac muscle structure. **C**. Cardiac muscle fibers.The discs contain desmosomes and gap junction (Cardiac Tissue Engineering: Principles, Materials, and Applications; Ruvinov, Sapir, Cohen, 2012)

Furthermore, the heart is composed of fibloblasts and endothelial cells. Cardiomyocytes are controlled by the involuntary nervous system. The myocardium assumes a unique structures, each fibre contains multiple cross-banded strands (called myofibrils), that fill up most of the cytoplasmatic space of each cardiac myocite. Each myofribil is formed by a lot of smaller contractile repeating structure: the sarcomere (Fig.3). Each sarcomere is defined as the structures bounded on each end by Z-disc, it is the site of polarity reversal of the actin filaments (thin filaments), as needed to interact with the bipolar myosin (thick filaments) (Luther, 2009). Two giant polymer proteins, titin and nebulin/nebulette, like actin, also overlap within and form important parts of the Z-disc (Clark *et al.;* 2002) (Fig.3).

Nebulin runs along the thin filament and forms the template for thin filament assembly

(McElhinny *et al.;* 2003). A sarcomere is defined as the arrangement of contractile proteins that resides between two consecutive Z-disks along a myofibril. Actin filaments anchored on each face of a Z-disk extend for 1 mm toward the center of adjacent sarcomeres. Thick filaments of the protein myosin sit in the center of each sarcomere and extend toward the Z-disks at the ends of the sarcomere. The thick filaments are connected at their centers by a protein matrix referred to as the M-line (or M-disk). The 3MDa 1 lm long protein titin runs between the M-line and the Z-disc (Young *et al.;* 1998). The region of the sarcomere in which the myosin filaments reside is known as the A-band. The area between A-bands is known as the I-band; each I-band is bisected by a Z-line and is traversed by the actin thin filaments (Handbook of Cardiac Anatomy, Physiology, and Devices, Second Edition, Paul A. Iaizzo Editor, 2009).

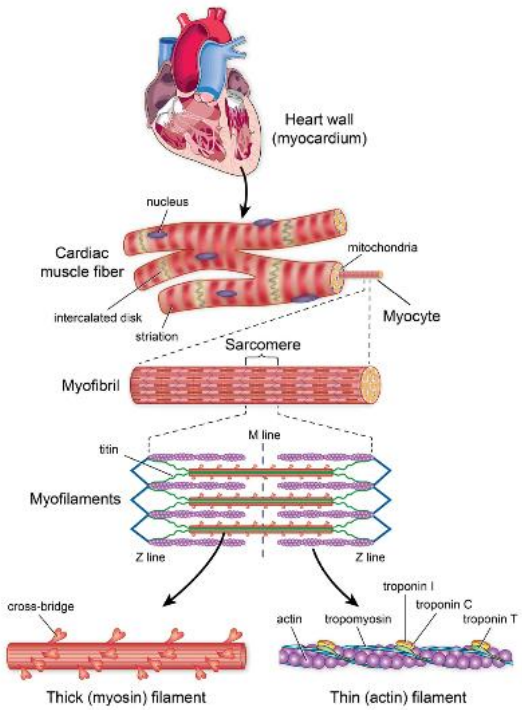


Figure 3. Striated muscle sarcomere showing its main components.Thick and thin filaments are organized into contractile units called sarcomeres. Thin filaments of the protein actin, a globular protein that is polymerized into a filament, are attached to the Z-line. Two regulatory proteins are found on the double-stranded actin thin filaments: tropomyosin and troponin: tropomyosin is a rod-shaped molecule that binds to actin and troponin; troponin, hinds myosin when it is bind to actin by fixing tropomyosin over the actin binding site (Golob *et al.;* 2014)

1.3.1 Thin Filament: Actin, Troponin and Tropomyosin

The principle structural component of the thin filaments is a double-stranded filament of the globular protein actin play a role in a lot of cellular processes like cell migration, cytokinesis, vescicle transport and contractile force generation (Pollard *et al.;* 2009). Actin in mature

striated myofibrils had been considered as a very stable structural component (Ono, 2010). The thin filaments also incorporate the regulatory proteins tropomyosin (Tm) and troponin (Tn). Tropomyosin is a α-helical coiled-coil that contains seven consensus pseudorepeats that binds to seven consecutive actin subunits along the long-pitch helix of actin filaments (Schmidt *et al.;* 2015). In skeletal and cardiac muscle, calcium binding to troponin causes a shift in tropomyosin's position along actin, exposing myosin binding sites and thereby facilitating muscle contraction (Lehman, Craig *et al.;* 1994).

Troponin is a globular protein complex with three subunits: TnC, a calcium-binding subunit; TnI, a subunit which facilitates inhibition of muscle contraction; and TnT, a subunit that connects the troponin complex to tropomyosin and actin (Barnett, 2009). Tropomyosin molecules are aligned end to end around the helical coil of the thin filament with one Tn complex attached to each Tm molecule. Troponin functions to couple Ca^{2+}concentration changes to azimuthal movement of tropomyosin on the thin filament. Tropomyosin's location on actin controls the access of cross-bridges to the thin filaments and thus regulates the cross-bridge cycling that drives contraction. In relaxed muscle, at low Ca^{2+}, tropomyosin is held by troponin at a location that sterically blocks the binding of the myosin heads to actin-binding sites (Yang *et al.;* 2014). During muscle activation and the subsequent increase in myoplasmic calcium concentrations, thin filaments are switched on when Ca^{2+} binds to troponin, than tropomyosin changes its position on the actin thin filament revealing the site on actin required for strong myosin binding (Fig.4). Myosin can bind the actine in a manner to create a forceand also alters the position of tropomyosin, and full activation of the thin filament requires binding of both calcium and myosin (Yang *et al.;* 2014) (Houmeida *et al.;* 2010).

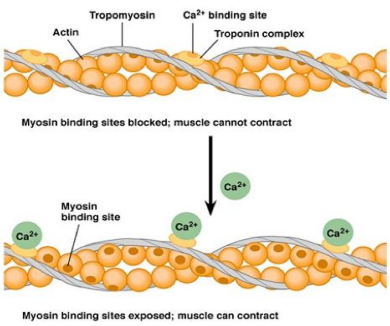


Figure 4. The troponin-tropomyosin complex. Tropomyosin and Troponin work together to block the myosin binding sites on actin. When a calcium ion bind to troponin, the troponin-tropomyosin complex moves, exposing myosin binding site

This thin filament-based mechanism for the regulation of contraction is also used for the control of skeletal muscle. In contrast, the activation of smooth muscle (the muscle of the vascular system, gut, and airways) is also calcium dependent. However in smooth muscle troponin is absent. The rise in calcium concentration is sensed by the cytosolic protein calmodulin and activation occurs via a different thick filament-based mechanism (Handbook of Cardiac Anatomy, Physiology, and Devices, Second Edition, Paul A. Iaizzo Editor, 2009).

1.3.2 Thick Filament: The Myosin

Myosins are a family of ATP-dependent molecular motor proteins responsible for force production and movement of muscle cells.

Myosin is best known for its role in muscle contraction. The protein is asymmetrically shaped with a long alpha-helical tail and two globular head domains. These head domains bind the actin and uses ATP hydrolysis to generate a force to walk along the filament of actin. The neckdomain acts as a linker for transducing force generated by the catalytic motor domain. The tail domain mediates interaction with other proteins and sometimes this domain play a role in regulating motor activity (Fig.5).

This results in a bipolar structure that has a bare zone in the center and the globular “head’’ domains of the myosin molecules projecting from each end (Handbook of Cardiac Anatomy, Physiology, and Devices, Second Edition, Paul A. Iaizzo Editor, 2009).

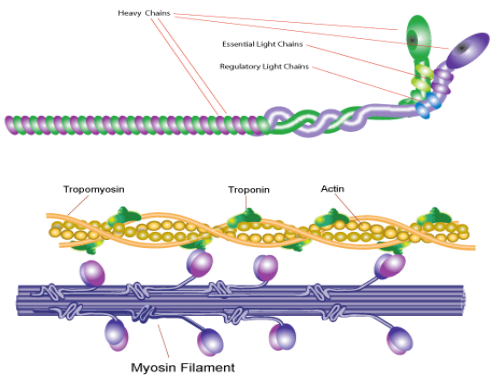


Figure 5. Myosin is the major contractile protein involved in eukaryotic muscle contraction by "walking" along actin microfilaments of the sarcomere

Myosins exist in different classes and the best known are:

Myosin I: ubiquitous cellular protein involved for the adaptation response of the stereocilia in the inner ear.

Myosin II: the myosin responsible for producing muscle contraction in muscle cells. It contains two heavy chains, which constitute the head and tail domains and contains 4 myosin light chains. In muscle cells, the long coiled-coil tails of the individual myosin join together, forming the thick filament of the sarcomere

Myosin III: it has been studied in vivo in the eyes of Drosophila Melanogaster where it is thought to play a role in phototransduction.

Myosin V: it is involved during the transport of cargo protein like vescicles, organelles, mitochondria, from the center of cell to the periphery.

Myosin VII: it is a plant-specific myosin linked to cell division.

Myosin XI: it directs the movement of organelles in plant cells.

1.4 THE CARDIAC ACTION POTENTIAL

The normal sequence and synchronous contraction of the atria and ventricles require the rapid activation of groups of cardiac cells. The generation of the action potential and the regional differences that are observed throughout the heart are the result of the selective permeability of ion channels distributed on the cell membrane (Grant, 2009).

Ion channel are fixed in the cell membrane and they have the ability to transport ions across the membrane. Their permeability to ions is very selective; each type of channel permits the passage of one or more specific ions. In excitable membranes, the three major classes of cation-selective ion channel are sodium, potassium and calcium. The permeability of these three channels is controlled by open/close gates in response to changes voltage potential. The potassium, sodium and calcium channels that contibute to the action potential in normal hearts are regulated by voltage-gated channels. At the resting membrane potential (-85 to -90 mV for most cardiomyocytes), the majority of potassium channels are in the open state, but sodium channels are closed (Milnor, 1990). Opening of the voltage-gated sodium channel in response to a less negative cytoplasm causes a high level of sodium into the cell and is the basis for the initiation of an action potential. When an excitatory stimulus causes the membrane potential to become less negative and further away a threshold level, the permeability of the membrane to ions changes, the cell rapidly depolarizes and the membrane potential reverses transiently prior to repolarization (Fig.6) (Cardiac Electrophysiology Methods and Models, D.Sigg, P.A. Iaizzo, Y.F Xiao, B. He, 2010).

Cell type	Resting membrane potential (mV)	Action potential duration (ms)	Conduction velocity (m/s)
Nerve			
Large myelinated	−80 to −90	0.2–1	100
Muscle			
Cardiac	−85 to −90	200–300	0.3–0.5
Skeletal	−80 to −90	1–5	3–5
Smooth	−50 to −60	10–50	

Figure 6. Major electrophysiological properties of excitable membranes.(Cardiac Electrophysiology Methods and Models, D.Sigg, P.A. Iaizzo, Y.F. Xiao, B. He, 2010)

The cardiac action potential is composed by 5 different phases showed below (Fig.7):

1. **Phase 4** is stable at ≈−90 mV in normal myocardial cells. The membrane potential at the onset of phase 4 is more depolarized (−50 to −65 mV), undergoes slow diastolic depolarization, and gradually merges into phase 0. This phase is called also "resting potential".

2. **Phase 0** is the phase of rapid depolarization. The membrane potential shifts into positive voltage range.

3. **Phase 1** is the phase of rapid repolarization. This phase detemines the potential for the next phase of the action potential. Epicardial cells have a prominent phase 1 and the shortest action potential.

4. **Phase 2** the plateau phase, is the longest phase. It is unique between excitable cells and marks the phase of cthe entry of calcium into the cell.

5. **Phase 3** is the phase of rapid repolarization that restoresthe membrane potential to its resting value.

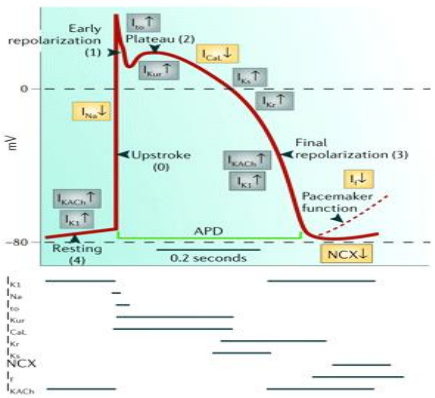


Figure 7. Action Potential: Resting (4), upstroke (0), early repolarization (1), plateau (2), and final repolarization. The action potential duration (APD) is approximately 200 ms (Grant *et al.;* 2009) (Nattel *et al.;* 2006)

The generation of the action potential and the regional differences that are observed throughout the heart are the result of the selective permeability of ion channels distributed on the cell membrane. The electrochemical gradient determines whether an ion moves into the cell (depolarizing current for cations) or out of the cell (repolarizing current for cations) (Fig.8). Homeostasis of the intracellular ion concentrations is maintained by active and coupled transport processes that are linked directly or indirectly to ATP hydrolysis (Grant, 2009).

Current	Selective ion	Current direction	Phase	Effect
I_{Na}	Na^+	Inward	0	Depolarization
$I_{Ca,T}$	Ca^{2+}	Inward	0	Depolarization
I_{to}	K^+	Outward	1	Early repolarization
$I_{Ca,L}$	Ca^{2+}	Inward	2	Plateau
I_{Kr}	K^+	Outward	3	Repolarization
I_{Ks}	K^+	Outward	3	Repolarization
I_{K1}	K^+	Outward	4	Resting potential
I_f	K^+/Na^+	Inward	4	Depolarization/ pacemaking

Figure 8. Major ion fluxes across cardiac plasma membrane during action potential (Cardiac Electrophysiology Methods and Models, D.Sigg, P.A. Iaizzo, Y.FXiao, B. He, 2010). Modified from Katz AM, Phisiology of the heart 2006, and Whalley DW, Basic concepts in cellular cardiac electrophysiology, 1995)

1.4.1 The Crossbridge Cycle

The interaction between myosin and actin in which the globular head of the myosin molecule bends towards and then binds to actin, contracts, releases actin, and then initiates a new cycle is known as myosin-actin cycling (Fig.9).

The myosin head domains contain an actin-binding site and an ATP hydrolysis site and hydrolyze ATP to ADP and phosphate (Pi). Electrical activation of the heart and contraction are coupled through the intracellular movement of calcium. Depolarization of the cardiomyocyte cell membrane during the action potential activates the L-type voltage-dependent calcium channels in the T tubule; the subsequent influx of calcium into the cell then leads to the opening of ryanodine-receptor channels inthe adjacent sarcoplasmic reticulum with a rapid increase in cytosolic calcium (Lopes *et al.;* 2016).

The binding of calcium to troponin removes the inhibition of the Tm–Tn complex on the thin filament. The energized myosin crossbridges can then bind to actin to the thin filament. This association with actin catalyzes the release of Pi and ADP, and a concomitant force-generating conformational change of the myosin head occurs while it is bound to actin.

The conformational change pulls the thin filament past the thick filament. At the end of the force-generating transition, myosin can rebind ATP, which reduces the affinity of the crossbridge for actin and causes cross-bridge detachment. The subsequent hydrolysis of the

myosin-bound ATP, in turn, reenergizes the crossbridge and prepares it for the next force-generating cycle. The cycle continues as long as the calcium concentration is high enough to keep the Tm–Tn complexes from blocking the myosin-binding sites (Handbook of Cardiac Anatomy, Physiology, and Devices, Second Edition, Paul A. Iaizzo Editor, 2009).

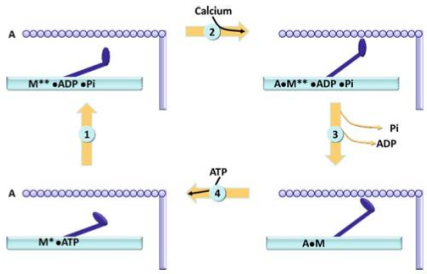


Figure 9. Crossbridge cycle: Step 1: Myosin (M) binds ATP on its globular head domain and hydrolyzes it to ADP and phosphate (Pi); Step 2: the energized crossbridge (M**ADP-Pi) binds to the actin thin filament. Step 3: The release of ATP hydrolysis products ADP and Pi from the crossbridge; Step 4: ATP binds to the M**ADP-Pi as it completes its powerstroke causing the dissociation of myosin and actin; Thecrossbridge cycle continues as long as the intracellular calcium concentration is high Handbook of Cardiac Anatomy, Physiology, and DevicesSecond Edition, Paul A. Iaizzo Editor, 2009)

1.5 TITIN

A lot of proteins of the sarcomeric cytoskeleton are organised by specific interactions with the giant protein of the sarcomere, the titin, encoded by the *TTN* gene. Titin is the biggest protein of the human body (over 1.5 μm) (Kontrogianni-Konstantopoulos *et al.;* 2009). The *TTN* gene consists of 364 exons, located on chromosome 2q31, that produces maximally a 4,200-kDa protein which is composed of ~38,000 amino acid residues. The size and complex structure of the TTN protein allows architectural support and preserves the sarcomeric organization during contraction, and developing passive tension during muscle stretching (Chaveau *et al.;* 2014). It also has a sensory and signaling role through the multiple TTN-binding proteins that are organized in signaling hot spots (Gigli*et al.;* 2016). Single titin molecules extend above the entire half-sarcomeres (from Z-disc to M-lines, more than 1 mm in length) and contain strain-compliant (Granzier *et al.;* 2005). The protein is organized in four structural and functional regions: the N-terminal Z-line (anchor to the sarcomeric Z-disk), the I-band (responsible for elastic properties), A-band regions (with a stabilizer role of the thick filament), and the C-terminal M-line extremity (overlap in antiparallel orientation with another C-terminal TTN molecule; modulation of TTN expression and turnover with the tyrosine kinase domain) (Chaveau *et al.;* 2014) (Fig.10).

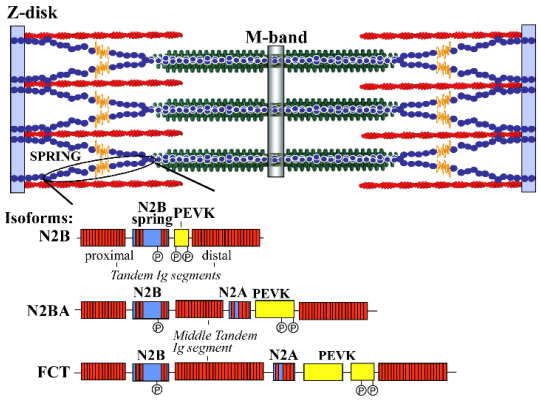


Figure 10. Domain structure of titin isoforms (LeWinter *et al.;* 2010)

The ~1.0 MDa region in the I-band consists of some immunoglobulin (Ig)-like domains in tandem that make up proximal (near Z-disk) and distal (near A-I junction) segments, interspersed by the PEVK sequence (rich in proline, glutamate, valine, and lysine residues) and the N2B element. The C-terminal ~2 MDa of titin is located in the A-band and is inextensible. It is composed of regular arrays of Ig and fibronectin type 3 (Fn3) modules forming the structures called so-called super-repeats.

A-band titin may function as a molecular ruler, regulating assembly of the thick filament. Titin's ~250-kDa COOH-terminal region is an integral part of the M-band and contains a kinase domain (LeWinter *et al.;* 2010).

The 364 exons of TTN undergo extensive alternative splicing to encode different isoforms. In cardiomyocytes, three different isoforms of titin are expressed: adult N2BA, adult N2B, and the fetal cardiac titin (FCT) isoforms.

Multiple splice pathways in the I-band encoding region (~230 exons) cause the different proprieties of each isoform (LeWinter *et al.*; 2010). The first isoform is the relatively small ~3.0 MDa isoform and it is known as N2B titin (it contains the N2B element).

A second class also contains the N2A element, and is named N2BA titin. N2BA contains additional spring elements in the PEVK and tandem Ig regions and resultsin a ~3.3-3.5 MDa size.

Due to the longer extensible I-band region, the N2BA titin isoform is more compliant than N2B titin (Fig.9). The ratio between these two isoforms is a major determinant of the cardiomyocyte stiffness (Hidalgo *et al.;* 2013) (Gigli *et al.;* 2016).

The third isoform predominates in fetal-neonatal life, which contain additional elements in both tandem Ig and PEVK regions, resulting in a ~3.6-3.8 MDa size protein. These isoforms

gradually disappear during postnatal development (LeWinter *et al.;* 2010).
The isoforms, N2BA and N2B are expressed 30-40 and 60-70% respectively, within the TTN protein in healthy adult human heart (Lahmers, 2004). A lot of different proteins bind the titin (Fig.11). Two N-terminal domains (Z1 and Z2) bind to small ankyrin-1 (sANK-1), a 17-kDa sarcoplasmic reticulum (SR) transmembrane protein (Kontrogianni-Konstantopoulos*et al.;* 2003).
This interaction plays a role in organizing the sarcoplasmic reticulum around the contractile apparatus at the Z-disk.
Furthermore, Z1 and Z2 interact with Tcap (telethonin), which assembles titin filaments into a tightly packed anti-parallel sandwich structure that is resistant to stretch (Zou*et al.;* 2006).
Additional Z-disk strength is provided by titin's Z-repeats, 45-amino-acid repeats that bind α-actininTcap also interacts with the potassium-channel subunit MinK found in T-tubules, which may modulate stretch-sensitive channel function (Sorimachi*et al.;* 1997) (Furukawa *et al.;* 2001) (LeWinter *et al.;* 2010).

1.5.1 Titin-Binding Protein

During the last years a variety of titin-binding proteins have been discovered.
Two different proteins bind the N2B site, one is αB-crystallin, a member of the small heat shock protein family that functions as a molecular chaperone (Bullard *et al.;* 2004). A newly identified group of LIM proteins characterized by 4 complete LIM domains and an N-terminal half LIM domain, FHL proteins, interacts also with N2B element (Sheikh *et al.;* 2008).
FHL-1 is found in cardiac and skeletal muscle and FHL-2 mainly in myocardium (LeWinter *et al.;* 2010). Additionally, FHL-1 interacts with members of the MAPK signaling pathways (Raf1, MEK1/2, and ERK2) that co-localize with N2B in the sarcomere (Sheikh *et al.;* 2008).
Together, these findings suggest that N2B facilitates assembly of a signaling complex that triggers hypertrophy in response to non-physiological N2B strain (LeWinter *et al.;* 2010).
Additionally, the N2A element binds to three homologous muscle ankyrin repeat proteins (MARPs): CARP, ankrd2, and DARP. MARPs participate in stress-activated pathways and are upregulated after mechanical or metabolic stimulus (Witt *et al.;* 2004). In the A-band, the first Ig domain of each 11-domain super-repeat interacts with myosin binding protein C (MYBPC3) (Freiburg *et al.;* 1996).
A-band titin should be a molecular ruler, which controls assembly and length of the thick

filament because it provides regularly spaced binding sites for myosin and MYBPC3 (LeWinter *et al.;* 2010).

The edge of titin's M-band region (A168-170) is a binding site for muscle specific ring finger protein (MURF) (Witt *et al.;* 2005).

MURF-1 is a sarcomere-associated protein that is an E3 ubiquitin ligase that conjugates ubiquitin to proteins destined for proteolysis.

The middle of M-line titin binds FHL-2 protein. Furthermore, the protein P94 binds the C-terminus site of titin (Kinbara *et al.;* 1997).

At last, the most C-terminal domain of titincontains a binding sites for obscurin, which is important for M-band stability.

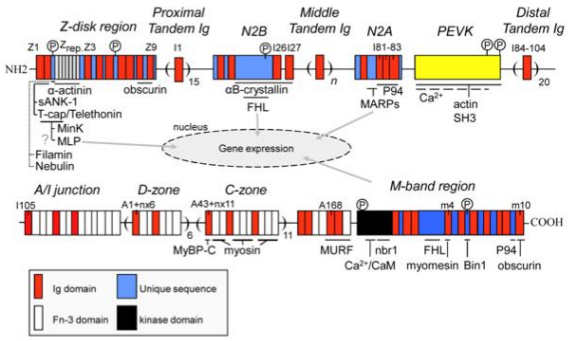


Figure 11. Proteins that bind to titin (Gigli *et al.;* 2016)

1.6 CARDIOMYOPATHY: AN OVERVIEW

Cardiomyopathy refers to an important and heterogeneous group of related diseases of the heart muscle in which the myocardium is structurally and functionally abnormal such as hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic right or left ventricular cardiomyopathy (ARVC/ALVC) and restrictive cardiomyopathy (RCM). Each phenotype is sub-classified into familial and non-familial forms. These conditions have many causes, symptoms, and treatments but the common complications can include mechanical and/or electrical dysfunction, heart failure and sudden cardiac death (SCD). In particular, undiagnosed disease (particularly in HCM and ARVC) is the major reason for SCD in young adults and athletes (Maron *et al.;* 2006) (Elliott *et al.;* 2008) (Maron *et al.;* 1996). It is possible to divided cardiomyopathies into two big groups based on predominant organ involved: primary cardiomyopathies and secondary cardiomyopathies. Primary cardiomyopathies (genetic, non genetic and acquired) are principally characterized by

problems connected to heart muscle (Fig.12); secondary cardiomyopathies show pathological myocardial involvement as part of a large number and variety of systemic multiorgan disorders (Tab.1) (Maron *et al.;* 2006).

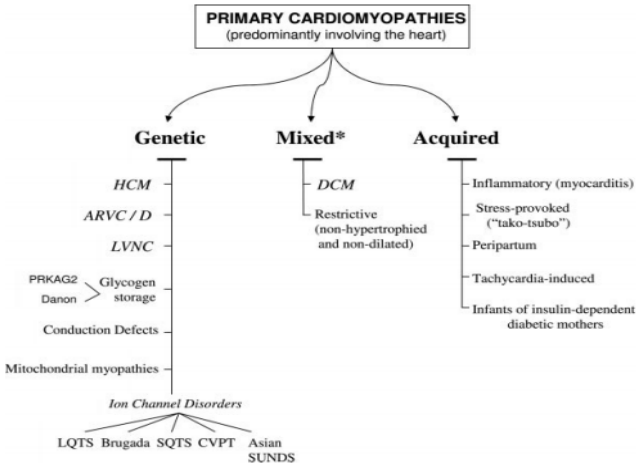


Figure 12. Classification of primary cardiomyopathies (Maron *et al*.; 2006)

Secondary Cardiomyopathies
Infiltrative*
Amyloidosis (primary, familial autosomal dominant†, senile, secondary forms)
Gaucher disease†
Hurler's disease†
Hunter's disease†
Storage‡
Hemochromatosis
Fabry's disease†
Glycogen storage disease† (type II, Pompe)
Niemann-Pick disease†
Toxicity
Drugs, heavy metals, chemical agents
Endomyocardial
Endomyocardial fibrosis
Hypereosinophilic syndrome (Löeffler's endocarditis)
Inflammatory (granulomatous)
Sarcoidosis
Endocrine
Diabetes mellitus†
Hyperthyroidism
Hypothyroidism
Hyperparathyroidism
Pheochromocytoma
Acromegaly
Cardiofacial
Noonan syndrome†
Lentiginosis†
Neuromuscular/neurological
Friedreich's ataxia†
Duchenne-Becker muscular dystrophy†
Emery-Dreifuss muscular dystrophy†
Myotonic dystrophy†
Neurofibromatosis†
Tuberous sclerosis†
Nutritional deficiencies
Beriberi (thiamine), pallagra, scurvy, selenium, carnitine, kwashiorkor
Autoimmune/collagen
Systemic lupus erythematosis
Dermatomyositis
Rheumatoid arthritis
Scleroderma
Polyarteritis nodosa
Electrolyte imbalance
Consequence of cancer therapy
Anthracyclines: doxorubicin (adriamycin), daunorubicin
Cyclophosphamide
Radiation
*Accumulation of abnormal substances between myocytes (ie, extracellular). †Genetic (familial) origin. ‡Accumulation of abnormal substances within myocytes (ie, intracellular).

Table 1. The most important common secondary cardiomyopathies (Maron *et al.;* 2006)

It is possible to divided cardiomyophaties not only in primary and secondary form, but in familial and non familial cardiomyophaties (Fig.13). The term familial concerns to a hereditary disease passes on from one generation to the next that could be caused by the same genetic mutation. Many familial cardiomyopathies are monogenic disorders and they can be sporadic when the causative mutation happens in an individual (*de novo* mutation) for the first time and it is not correlated with the family (Elliott *et al.;* 2008). Non-familial cardiomyopathies are clinically defined by the presence of a cardiomyopathy in a patient and the absence of disease in other family members. Futhermore some of these disease do not have a uniformly expression and may evolve from one category to another during their natural clinical course (Maron *et al.;* 2006)

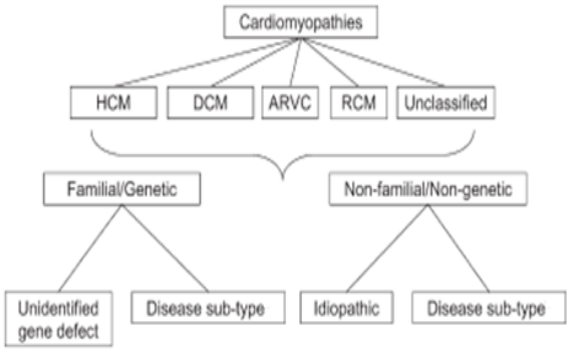


Figure 13. Classification of familial and non-familial cardiomyophaties (Elliott *et al.*; 2008)

The panel of the American HeartAssociation (www.heart.org) has suggested that ion channelopathies and disorders of conduction should also be considered as cardiomyopathies. The Association declares that these genetic cardiovascular disorders are responsible for altering biophysical properties and protein structure with the effect of create a structural abnormal ion channel interfaces and architecture (Maron *et al.*; 2006). Corcern to these disorders, it is particularly relevant to establish a correct medical evaluation of the affects and their family history (Morales *et al.;* 2008). The first goal of family history is to know if the cardiomyopathy is familial, ad, if so, to identify those individuals who may be at risk. Indeed, practice on clinical management and genetic testing for inherited cardiomyopathies recommend taking a detailed family history that includes at least three generations for the reduced penetrance observed in some families with cardiomyopathy, clinically screening at-risk family members, counseling patients on a possible inherited cause, and considering genetic testing of the most clearly affected person in the family (Ackerman *et al.;* 2011) (Hershberger *et al.;* 2009). Genetic testing of at-risk family members can remove the disease

risk (when negative) or identify those in need of clinical monitoring or intervention to decrease the risk of morbidity or mortality (when positive). However, because genetic testing for inherited cardiomyopathies is a relatively young discipline, the spectrum of pathogenic variants present in the population is incompletely characterized, even in well-established disease genes (Teekakirikul *et al.;* 2013). The evidence of genetic and phenotypic overlap among different cardiomyopathies adds other complexity (Fig.14), often resulting in stepwise analysis of multiple disease-specific genes when the diagnosis is not so clear. For example, several sarcomeric gene mutations cause both DCM and HCM and troponin I is mutated in hypertrophic cardiomyopathy and restrictive form of cardiomyopathy (Maron *et al.;* 2006). Improved next generation sequencing (NGS) technologies have resulted in a remarkable increase in speed and efficiency about cardiovascular genetic testing of a large number of gene simultaneously, which empowers the practice of cardiovascular genetic medicine (Hershberger *et al*.; 2013).

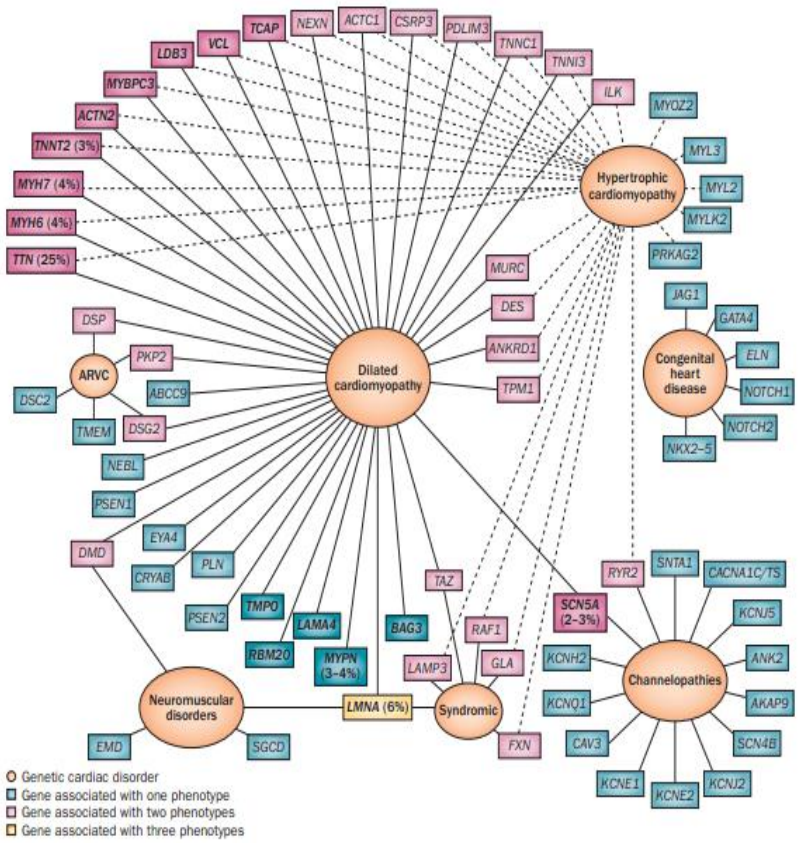


Figure 14. Relationship between genes associated with cardiomyopathies and related phenotypes (Hershberger *et al.;* 2013)

1.7 HYPERTROPHIC CARDIOMYOPATHY: DIAGNOSIS, MANAGEMENT AND TREATMENT

Hypertrophic cardiomyopathy is the most common genetic cardiovascular disorder, affecting one of every 500 adultsand it is inherited in an autosomal dominant Mendelian pattern with variable expressivity and age-related penetrance (Maron *et al.;* 2002) (Ho, 2010a). Offspring of an affected individual have a 50% probability of inheriting a mutation and risk for disease; alternatively, sporadic cases may be due to *de novo* mutations in the proband but absent from the parents (Maron *et al.;* 2012). Hypertrophic cardiomyopathy affects people of both genders and of various racial and ethnic origins and it is the most common cause of sudden death in young athletes in US (Teekakirikul *et al.;* 2012) (Maron *et al.,* 1996) (Ho, 2010a). Hypertrophic cardiomyopathy is a primary genetic cardiomyopathy (Maron, 2006) characterized by left ventricular hypertrophy (LVH) which is usually asymmetric, with greater involvement of the interventricular septum than the left ventricular-free wall (Fig.15A-B) in the abscence of another systemic or cardiac process (Teekakirikul *et al.;* 2012). Since the first description of hypertrophic cardiomyopathy (HCM) as a clinical entity in 1958, the disease has interestedmostlyboth clinicians and researchers for its heterogeneity in clinical, phenotypic expressions a variable natural progression, wich may range from dyspnea and/or syncope to sudden cardiac death (Baxi *et al*.; 2016). Heart muscle changes affect the electrical stability of the myocardial cells, predisposing to heart failure and/or arrhythmias (Dische, 1972). LVH is caused by an increase in myocyte size, but not myocyte number, and by a greater amount of myocardial fibrosis, which is distributed throughout the interstium and in discrete foci. HCM myocytes are characterized by distorted nuclei, disorganized myofibrils, and abnormal registration of adjoining myocytes, which combined with increased fibrosis, contributes to a characteristic cardiac histopathology (Fig. 15C-D).

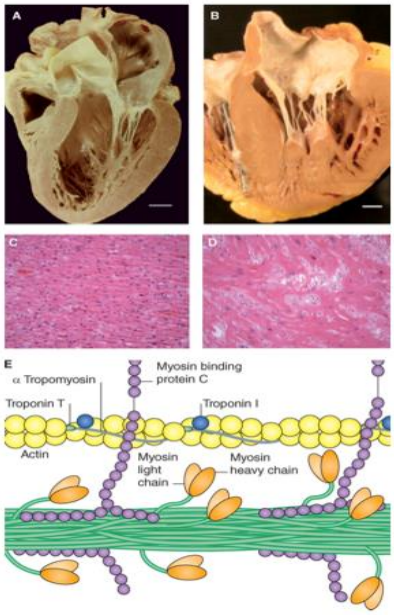


Figure 15. Cardiac manifestation of HCM**. A.** The normal hearth, **B.** The HCM hearth with increased thickness of the walls and papillary muscle (Image A is reprinted from *Wang et al.;* 2010). **C.** The normal myocardial hystology, **D.** HCM shows misaligned nyocytes with enlarged nuclei and expanded interstitial matrix (light pink). **E.** Major contractile proteins involved in HCM (Teekakirikul *et al.;* 2012)

Experimental data suggest that cardiac myocyte contractile function in HCM is reduced and that the hypertrophy is compensatory (Marian 2000) (Rust *et al.;* 1999). These report, in conjunction with myocyte disarray led to the hypothesis that tissue Doppler imaging (TDI) with its possibility to identify contraction and relaxation abnormalities would be more sensitive for the diagnosis of HCM than conventional echocardiography. This principle has provided by several studies in both animals and patients carrying different sarcomeric mutations (Nagueh *et al.;* 2000) (Nagueh *et al.;* 2001) (Ho *et al.;* 2002). Up to 70% of patients with HCM display the left ventricular outflow tract obstruction (LVOT) strongly associated with progression to severe symptoms of heart failure and of death (Maron *et al.;* 2003). This obstruction is induced by thickening of the interventricular septum (IVS) and systolic anterior movement (SAM) of the mitral valve. Furthermore, hypertrophic cardiomyopathy can be associated with intrinsic abnormalities of the mitral valve, including increased mitral leaflet area, length, and laxity, as well as anterior displacement of the papillary muscles, which may predispose to residual systolic anterior motionand result in suboptimal outcome after isolated myectomy (Fig.16) (Dearani *et al.;* 2007) (Heric *et al.;* 1995)

In seriously symptomatic patients with obstructive HCM, despite optimal medical therapy,

isolated myectomy is the golden standard with excellent perioperative and long-term outcomes (Woo *et al.;* 2005) (Maron *et al.;* 2003) (Ommen *et al.;* 2005).

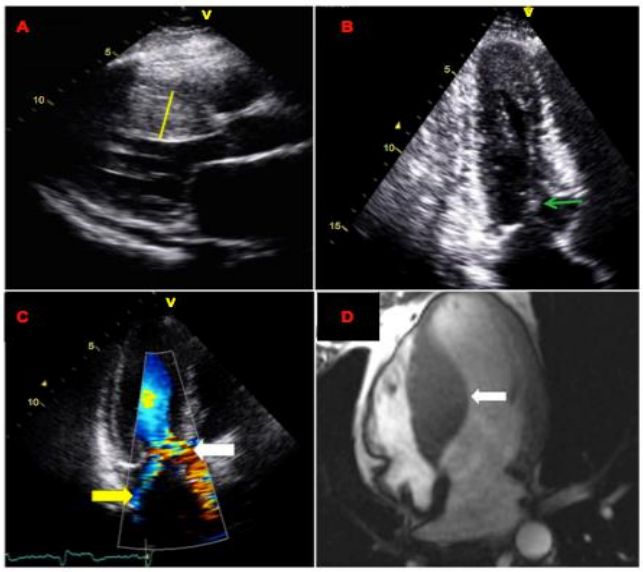


Figure 16. Imaging of HCM. **A.** Transthoracic echocardiogram in the parasternal long axis view showing basal septal hypertrophy (yellow line). **B.** Transthoracic echocardiogram in the apical view showing basal septal hypertrophy and SAM of the mitral valve (green arrow). **C.** Transthoracic echocardiogram in the apical view with color-flow Doppler showing turbulent flow in the outflow tract (white arrow) and mitral regurgitation because of mitral valve SAM. **D.** Cardiac MRI in the apical view showing striking asymmetric basal septal hypertrophy (white arrow). All images are provided by Dr. Theodore Abraham, Johns Hopkins Hospital (Houston, 2015)

Based on more recent, balanced overviews of patients with HCM, the annual mortality for patients with HCM is estimated at 1% per year (Elliott *et al.;* 2014) (Maron *et al.;* 2002) (Maron *et al.;* 2013).

The clinical heterogeneity of HCM needs for individual treatment:

(1) Symptom management

(2) risk stratification for sudden cardiac death

(3) counselling/screening, including exercise and lifestyle recommendations, family screening, and genetic counseling (Fig.17)

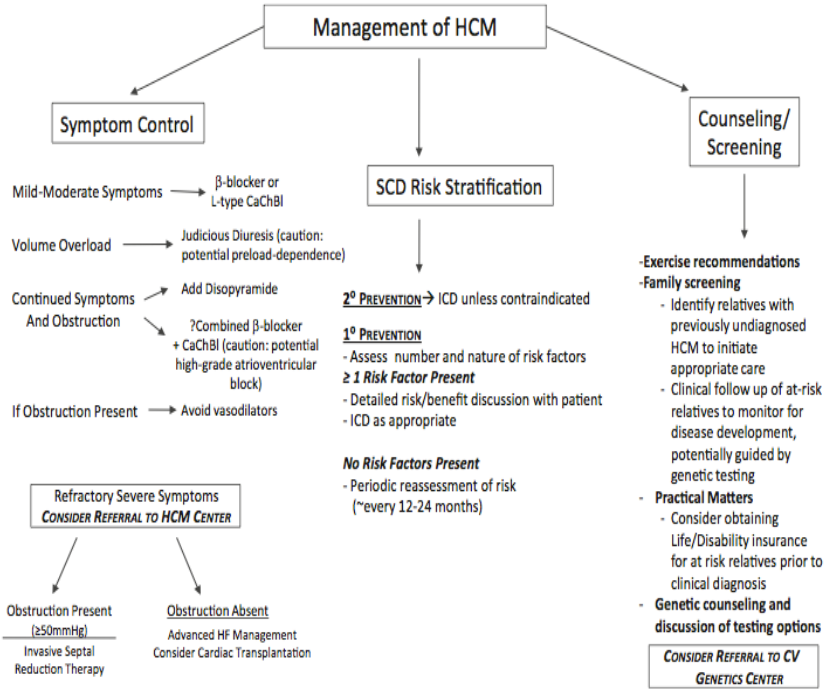


Figure 17. Clinical management of hypertrophic cardiomyopathy (HCM). CaChBl indicates calcium channel blocker; SCD, sudden cardiac death; HR, heart rate; ICD, implantable cardioverter-defibrillator; HF, heart failure; and CV, cardiovascular (Ho *et al.;* 2012b)

Management in patients affected by hypertrophic cardiomyopathy is direct towards control of symptoms, risk stratification and prevention for SCD, screening of relatives and the correct theraphy (Tab. 2)

Functional consequence	Clinical manifestations	Options for pharmacotherapy
Diastolic dysfunction	• Dyspnoea • Reduced exercise capacity	• β-Blockers, regardless of whether there is coexisting LVOTO • Nondihydropyridine calcium-channel blockers (verapamil or diltiazem), used with caution in the presence of significant LVOT gradient
LVOTO, mitral regurgitation	• Chest pain • Dyspnoea • Reduced exercise capacity • Symptoms of impaired consciousness	• Resting LVOT gradient ≥30 mmHg is a predictor of both all-cause mortality and arrhythmic events, but whether pharmacotherapy reduces this risk is currently unresolved. The incidence of SCD in asymptomatic individuals with resting LVOTO as their sole risk factor is estimated at <0.4%, arguing against intervention for prognostic reasons. Treatment is, therefore, directed towards symptom relief • General advice is to ensure adequate filling and avoid vasodilators such as amlodipine and positive inotropic agents such as digoxin • Nonvasodilating β-blockers are used as first-line therapy and are particularly effective for exertional LVOTO • Disopyramide is used as an add-on to β-blockers, particularly for symptomatic resting LVOTO; dose is titrated according to tolerance of anticholinergic adverse effects. Monitoring of QT interval is advised, with avoidance of other QT-prolonging agents (for example, amiodarone and sotalol) • Disopyramide plus verapamil/diltiazem can be used if β-blockers are contraindicated • Disopyramide should not be used as monotherapy in the presence of atrial fibrillation because it might accelerate the ventricular response rate • Verapamil/diltiazem can also be used as monotherapy if β-blockers are contraindicated, but caution is warranted, particularly in the presence of severe LVOTO • In the rare setting of hypotension or pulmonary oedema secondary to acute LVOTO, patients who do not respond adequately to filling might benefit from intravenous β-blockers and/or vasoconstrictors such as phenylephrine • Invasive septal reduction is an option for patients with NYHA class III–IV symptoms despite maximum tolerated pharmacotherapy
Ischaemia	• Chest pain • Dyspnoea • >2 mm ST-segment depression on exercise (usually far deeper) • Reversible defect on perfusion scan	• If obstructive coronary artery disease is unlikely or has been excluded, microvascular ischaemia can be presumed • Nondihydropyridine calcium-channel blockers such as verapamil or diltiazem are often used first line in this setting; β-blockers are an alternative
Atrial fibrillation	• Palpitation • Dyspnoea • Presyncope/syncope	• Consider anticoagulation (CHA_2DS_2-VASc score not recommended for use in patients with HCM) • Rhythm control with sotalol or amiodarone (with variable success) • Rate control with β-blockers or nondihydropyridine calcium-channel blockers
Ventricular arrhythmia	• Palpitation • Presyncope/syncope	• Nonsustained VT on ambulatory ECG monitoring is a risk factor for SCD; amiodarone plus β-blockers might suppress ventricular extrasystoles and nonsustained VT, and appeared, in studies from the pre-ICD era, to confer a survival benefit. In contemporary practice, ICD therapy is first line for individuals with a high-risk profile • Sustained VT is uncommon and warrants evaluation for coexisting coronary artery disease and/or LV apical aneurysm with adjacent scarring. ICD is indicated for prevention of SCD; β-blockers and/or amiodarone are often of benefit in suppressing arrhythmia
Abnormal vascular responses	• Symptoms of impaired consciousness • Failure of systolic BP to rise by ≥20 mmHg during maximal upright exercise testing or fall >20 mmHg from peak pressure	• The selective serotonin reuptake inhibitor paroxetine has been shown to reverse paradoxical vasodilatation, alleviate associated symptoms, and augment systolic BP in patients with HCM with an abnormal BP response to upright exercise • Pharmacological therapy for vascular instability has the potential to be of both symptomatic and risk-modifying benefit, but remains largely empirical until further data become available
Wall thinning and cavity dilatation	• Symptoms of heart failure	• Conventional therapy for systolic heart failure (including angiotensin-converting-enzyme inhibitors, angiotensin-receptor blockers, β-blockers, and diuretics if necessary)

Table 2. Clinical manifestations of HCM and their relative theraphy. BP, blood pressure; ECG, electrocardiogram; HCM, hypertrophic cardiomyopathy; ICD, implantable cardioverter-defibrillator; LV, left ventricular; LVOT, left ventricular outflow tract; LVOTO, left ventricular outflow tract obstruction; SCD, sudden cardiac death; VT, ventricular tachycardia (Sen-Chowdhry *et al.;* 2016)

Prophylactic pharmacological treatment in with no clinical symptoms has not been proved to be effective in preventing progression of disease. Beta-blockers decrease the heart rate wich results in a prolongation of the diastole and relaxation phase of the heart and an increase in passive ventricular filling. The use of Verapilim, a calcium antagonist drug, also has favourable effects on symptoms by improving ventricular relaxation and filling. Disopyramide, a negative intropic and type I-A antiarrhythmic drug, is used in patients with progressive heart failure symptoms with left ventricular outflow tract obstruction and it can decrease systolic anterior movement of the mitral valve, LVOTO, and mitral regurgitation (Sen-Chowdhry *et al.;* 2016). In patients with hypertrophic cardiomyopathy, attention to specific lifestyle changes can help mitigate symptoms and may reduce the risk of sudden cardiac death, for example, even moderately intense physical activity may lead to syncope or SCD. During exercise cardiac inotropy and chronotropy augmented and the systemic vascular

resistance (SVR) decreases without the ability to augment cardiac output because of outflow obstruction resulting in systemic hypoperfusion. It is recommended that patients with HCM should not participate in most competitive sports with the possible exception of those that involve low intensity (Houston, 2015) (Elliott *et al.*; 2014).

1.7.1 Molecular genetic basis of hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy is the most common monogenic cardiovascular disordeswith variable expressivity and penetrance, affected one of every 500 adults inherited like an autosomal dominant trait (Maron *et al.;* 1995). Jarcho and colleagues identified the genetic locus responsible for familial HCM in 1989 used linkage analysis of a large, multigenerational family to identify a portion of the long arm of chromosome 14, which cosegregated with incidence of disease (Jarcho *et al.;* 1989). The following year, Geisterfer-Lowrance et al. identified the first HCM-causative mutation in the *MYH7*-encoded beta myosin heavy chain (Geisterfer-Lowrance *et al.;* 1990). Consequently, hypertrophic cardiomyopathy became the first cardiac disease for which a molecular genetic cause was determined (Ho *et al.;* 2015). Since the identification of the first mutation in *MYH7*, hypertrophic cardiomyopathy has been viewed like a disease of the sarcomere, with the typical molecular characteristics considered to result from inadequate or dysregulated force generation (Seidman *et al.;* 2001). More than 1500 mutations in any of at least 11 genes encoding the molecular components of the sarcomere can lead to development of HCM, with the majority of these found in one or only in a few families (Seidman *et al.;* 2011) (Ho, 2010b) (Baxi *et al.;* 2016). One or more of these genetic mutations are found in up to 60% of individuals with a family history of HCM and 30% of those without a family history (Gersh *et al.;* 2011). Combination of more than one mutation is not rare in 6% of affected individuals (Ingles *et al.;* 2013). Mutations can be located in many genes, but the most is possible to find in the genes encoding sarcomeric proteins (Seidman *et al.;* 2011) (Tab.3) (Fig.18). The most commonly affected genes identified include *MYBPC3* (myosin binding protein C, 11p11.2, 30%-40%), *MYH7* (myosin heavy chain 7, 14q11.2, 20%-30%), *TNNT2* (cardiac muscle troponin, 1q32.1, 1'%), *TNNI3* (troponin I type, 19q13.42, 7%), *MYL2* (myosin light chain 2, 12q24.11, 4%), *MYL3* (myosin light chain 3, 3p21.31, 2%) and *TPM1* (tropomyosin 1, 15q.22.2, 1%). The resulting pathogenetic mutations might result in exchange of a single amino acid for another at the protein level, or result in more radical

truncation of a protein with insertion or deletion nucleotides.

	Gene	Location	Frequency (%)
Prevalent sarcomeric genes			
Thick filament			
β-Myosin heavy chain	*MYH7*	14q11.2	20–30
Regulatory myosin light chain	*MYL2*	12q23-q24	2–4
Essential myosin light chain	*MYL3*	3p21.3	1–2
Thin filament			
Cardiac troponin T	*TNNT2*	1q32.1	10
Cardiac troponin I	*TNNI3*	19q13.4	7
α-Tropomyosin	*TPM1*	15q22.1	<1
α-Cardiac actin	*ACTC1*	15q11q14	<1
Intermediate filament			
Cardiac myosin-binding protein C	*MYBPC3*	11p11.2	30–40
Rare sarcomeric and Z-disc genes[a]			
α-Actinin 2	*ACTN2*	1q43	<1
α-Myosin heavy chain	*MYH6*	14q11.2	Rare
Muscle LIM protein	*CSRP3*	11p15.1	Rare
Telethonin	*TCAP*	17q12	Rare
Calcium handling or regulation genes[a]			
Phospholamban	PLN	6q22.3	Rare
Calsequestrin	CASQ2	1p13.1	Rare
Junctophilin 2	JPH2	20q13.12	Rare
Non-sarcomeric genes associated with cardiac hypertrophy	Gene	Associated phenotypes	Transmission/Frequency
Protein kinase, AMP-activated, gamma 2 subunit	*PRKAG2*	Wolff–Parkinson–White syndrome	Dominant/rare
Lysosomal-associated membrane protein 2	*LAMP2*	Danon disease	Dominant/rare
Galactosidase, alpha	*GLA*	Fabry	X Linked/1–2% of males
Four and a half LIM domains 1	*FHL1*	FHL1-related diseases	X Linked/rare
Transthyretin	*TTR*	Amylose	Dominant
Glucosidase, alpha	*GAA*	Pompe	Recessive/rare
Protein tyrosine phosphatase, non-receptor type 11	*PTPN11*	Noonan	Dominant/rare
Frataxin	*FXN*	Friedreich	Recessive/rare

[a]For these genes, evidence for pathogenicity in HCM has not been clearly established.

Table 3. Genes associated with HCM (Ho *et al.;* 2015)

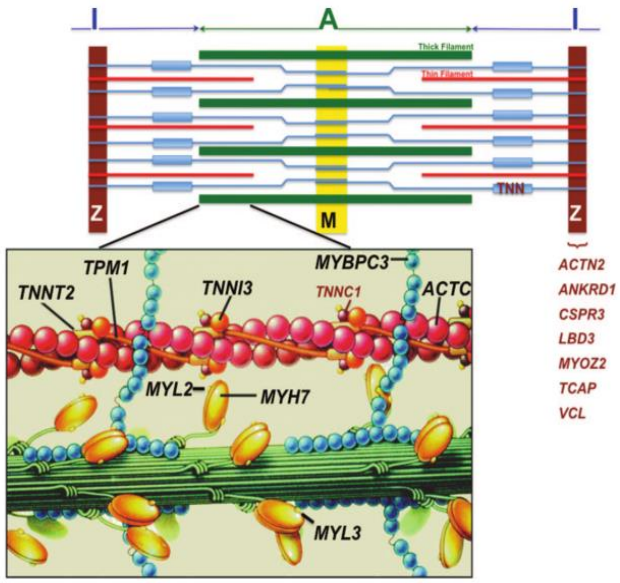


Figure 18. Main sarcomeric genes involved in HCM (Seidman *et al.;* 2011)

The two most frequently mutated genes are *MYBPC3* and *MYH7*, encoding the sarcomeric proteins cardiac myosin-binding protein C and beta myosin heavy chain (Christiaans *et al.;*

2010) (Charron *et al.;* 2006).

In particularly, *MYBPC3* mutations predicted to lead to more structural changes or truncation of the protein are frameshift mutations (mutations resulting in the insertion or deletion of ≥1 nucleotide in the coding region of genes), non-sense mutations leading to premature termination of translation, or mutations affecting normal splicing of mRNA (mutations involving the consensus splice sites), or intronic mutations leading to cryptic abnormal splice sites) (Ho *et al.;* 2015).

Furthermore, Mouton and colleagues affirm that it has recently been shown that MYBPH (myosin binding protein H, 1q32.1) functions with MYBPC3 in regulating cardiomyocyte contraction in response to β-adrenergic stress. Individual knockdown of either MYBPH or MYBPC3 had little effect on cardiomyocyte contraction (Mouton *et al.;* 2016).

Therefore, given the similarity between MYBPC3 and MYBPH in sequence homology and structure and the important role that MYBPH plays in cardiomyocyte contraction, Mouton and colleagues proposed that MYBPH, like MYBPC3, might be involved in HCM pathogenesis but, to date, no disease-causing mutation has been found in MYBPH. They hypothesized that variants in MYBPH may play a role in modifying the hypertrophic phenotype. Mutations in the troponins, cardiac troponin T and troponin I, and α-tropomyosin (*TPM1*), are also relatively common, a total of at least 69 mutations have been identified in cTn subunit proteins that have been reported to be associated with HCM, including 30 in troponin I, and 34 in troponin T (Cheng *et al*.; 2016). The majority of *TNNI3* mutations associated with the HCM phenotype are located in the C-terminal region. Almost 60% of the HCM-causing *TNNI3* mutations occur through the substitution of a positively or negatively charged amino acid for a neutral or hydrophobic one (Marques *et al.;* 2016). Of note, arginine replacement occurs ~40% of the time (Willott *et al.;* 2010). All of the mutations are single nucleotide polymorphisms (SNPs or variants), and the degree of cardiac pathology associated with them is highly variable as is verification of their causal nature (Cheng *et al.;* 2016). The HCM-associated mutations in the myosin ELC (product of *MYL3* gene) are quite rare, but they are also linked to malignant outcomes (Huang *et al.;* 2015). *De novo* mutations and germlinemosaicism occurs very rarely in HCM (Rai *et al.;* 2009) (Cuda *et al.;* 1996). A lot of mutations correlated to HCM are private, many of the identified mutations are novel. In certain countries/populations, however, founder mutations have been identified and the haplotype analysis suggests a common ancestor. Founder mutations have been found in the Netherlands (Alders *et al.;* 2003), South Africa (Moolman-Smook *et al.;* 1999), Finland (Jääskeläinen*et al.;* 2004), Italy (Girolami *et al.;* 2006), Japan (Kubo *et al.;* 2005) and in the

Amish population of the United States (Zahka *et al.;* 2008). These mutations most occur in *MYBPC3* and the majority encode a truncated protein and implies that these defects have far less deleterious consequences than other HCM mutations (Seidman *et al.;* 2011). Landstrom and colleagues report three missens mutations in *JPH2* (Junctophilin-2) as a novel genetic basis for HCM (Landstrom *et al.;* 2009). Junctophilin-2 is involved in the calcium signaling apparatus of cardiomyocytes and the discovery of mutations in that gene supports as a key role for calcium perturbations in a pathway that culminates in the clinically expressed disease phenotype of hypertrophic cardiomyopathy. Two to five percent of patients with HCM harbor two mutations (compound or double heterozygosity) or are homozygous for a mutation (Force *et al.;* 2010) (Marian *et al.;* 2008) (Ingles *et al.;* 2005) (Olivotto *et al.;* 2008). It appears that these patients may develop a more severe clinical phenotype (Force *et al.;* 2010). Hypertrophic cardiomyopathy associated with triple sarcomere gene mutations is rare but often associated with adverse outcomes, including an increased risk of developing end-stage disease progression and high prevalence of ventricular arrhythmias. These findings support the theory that multiple mutations develop more significant left ventricular hypertrophy and require more advanced and invasive treatments such as surgical myotomy/myectomy and AICD (automated implantable cardioverter-defibrillator) implantation (Ingles *et al.;* 2005)(Girolami *et al.;* 2010). Recent studies discovered the implication of several genes encoding components of the Z-disc (previously associated with dilated cardiomyopathy) in the pathogenesis of HCM like titin (*TTN,* 2q31.2), muscle LIM protein (*MLP*/*CSRP, 11p15.1*), telethonin (*TCAP, 17q12*) and myozenin 2 (*MYOZ2, 4q26*) (Alcalai *et al.;* 2008) (Hayashi *et al.;* 2004A) (Osio *et al.;* 2007) (Arad *et al.;* 2005) (Hayashi *et al.;* 2004b) (Geier *et al.;* 2003). However, for some of these genes, evidence for direct pathogenicity in HCM has not been clearly established. The considerable variability in penetrance and expressivity showed by individual mutations, not only across unrelated familiesbut even within the same family, indicate that factors other than the sarcomere mutation itself influence clinical course and outcomes. If a pathogenic mutation can be identified in a family, other relatives can undergo predictive genetic testing to findif they have inherited the mutation, before a clinical diagnosis can be established.

1.7.2 Cardiac titin mutations and hypertrophic cardiomyopathy

Titin is a giant sarcomeric protein of the human body (over 1.5 μm) that provides passive stiffness to cardiac myocites and stress sensitive signaling (Kontrogianni-Konstantopoulos *et al.;* 2009) (LeWinter *et al.;* 2014). The *TTN* gene consists of 364 exons, located on chromosome 2q31, that produces maximally a 4,200-kDa protein which is composed of ~38,000 amino acid residues. At its N-terminus, titin is fixed in the Z-disk of the sarcomere. The rest of the protein is divided into an elastic I-band region, a thick filament-binding A-band region and the M-band region where the C-terminus is embedded (LeWinter *et al.;* 2013) (Fig.19A, bottom).

The I-band region of titin is comprised of three elements:

1) tandem Ig segments consisting of serially-linked immunoglobulin (Ig)-like domains,

2) the PEVK segment (with high percentages of proline, glutamic acid, valine, and lysine),

3) the N2B element with its extensible unique sequence (N2B-Us) (Fig.19A, middle) (Labeit *et al.;* 1995) (Bang *et al.;* 2001) (LeWinter *et al.;* 2013).

This protein is encoded by a single gene but different mRNA splice pathways result in a distinct isoform classes. In cardiac muscle three classes are present: adult N2BA, adult N2B, and fetal cardiac titin (FCT) (Fig.19A middle) (Labeit *et al.;* 1995) (Bang *et al.;* 2001) (Greaser *et al.;* 2005) (LeWinter *et al.;* 2013). These classes differ in their I-band region; the rest of the molecule is largely identical. Adult cardiac muscle co-expresses N2B and N2BA titin at the level of the half-sarcomere (Trombitas *et al.;* 2001). Finally, the cardiac isoform novex-1 (2980kDa) and the cardiac and skeletal muscle isoform novex-2 (2980kDa) are nearly identical to N2B TTN, these two isoforms differ only in the incorporation of 125 or 192 amino acid (respectively) stretches in the I-band region. Novex-3 is presented in all striated muscle and is the only isoform expressing the 8-kb-novex-3 exon that introduces an alternative terminal coding exon (Chaveau *et al.;* 2014).

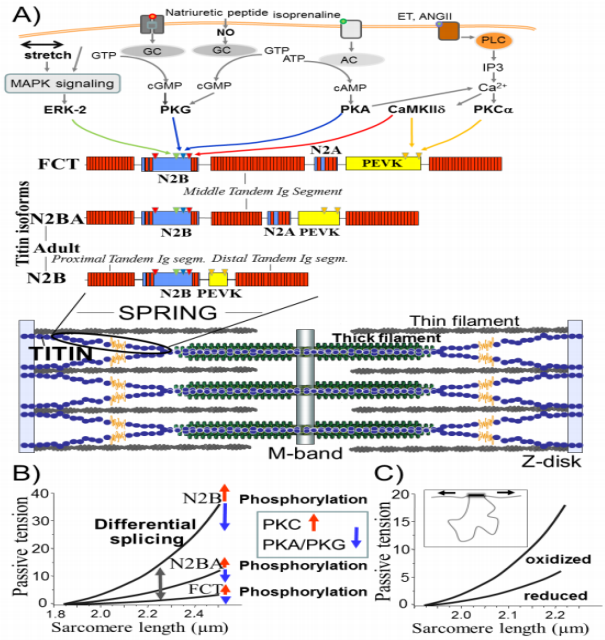


Figure 19. Structure of titin in the sarcomere (A) and mechanisms for modifying titin-based passive tension (B and C) **A.** Bottom: Single titin molecules (blue and yellow) span from Z-disk (N-terminus) to M-band (C-terminus). Middle: Composition of extensible I-band region of N2B, N2BA titin isoforms (found in adults) and fetal cardiac titin isoform (FCT). Red blocks are for theIg-like domains, blue, unique sequence and yellow, PEVK sequence. Top: Phosphorylation sites in titin's spring region (present in all 3 isoforms) and their upstream signaling pathways. **B.** Schematic of force-extension curves of titin isoforms and effects of phosphorylation on passive tension. **C.** Schematic effect of oxidation to form cysteine disulfide crosslinks in the N2B region of titin (inset) and effects on passive tension. MAPK: mitogen-activated protein kinase; GC: guanylylcyclase; AC: anelylylcyclase; NO: nitric oxide; ET: endothelin; ANGII: angiotensin II; IP3: inositol triphosphate (LeWinter *et al.;* 2013)

Most of the initially identified *TTN* mutations were associated with DCM (Fig.20)(LeWinter *et al.;* 2014). Altered titin isoform expression ratios have been reported in patients with heart disease, i.e. patients with ischemic cardiomyopathy express increased amounts of N2BA titin, accompanied by reduced myofibrillar stiffness (Neagoe *et al.;* 2002). Titin mutations are a rare in patients with hypertrophic cardiomyopathy but some *TTN* changes associated to HCM are distributed along the whole *TTN* sequence (Chaveau *et al.;* 2014) (Fig.21).

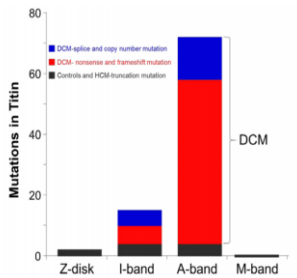


Figure 20. Distribution of titin mutations. DCM-causing mutations are absent from the Z-disk and M-band region and are most prominent into the A-band region (LeWinter *et al.;* 2013, figure based on data from Herman *et al.;* 2012)

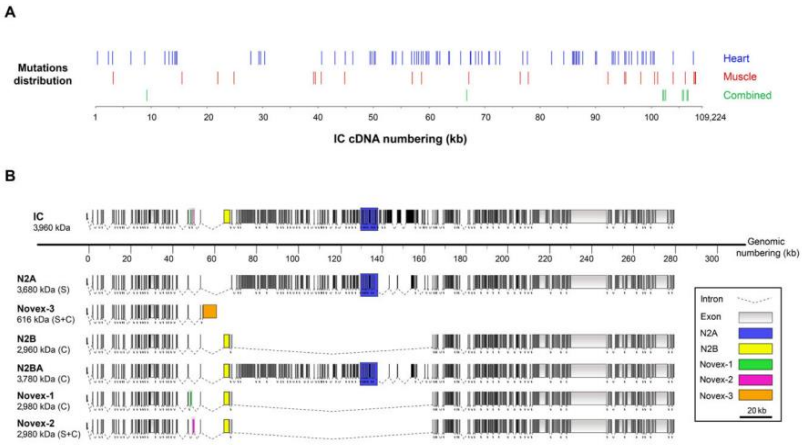


Figure 21. Distribution of the TTN variants reported along the gene. **A.** Approximative position and distribution of the known TTN mutation. **B.** Schematic representation of the exons encoding the main TTN isoforms . Variants related to purely cardiac phenotype are indicated in blue, purely skeletal muscle phenotypes in red and phenotypes associating with both cardiac and skeletal muscle in green (Chaveau *et al.;* 2014)

Only 4 titin missense mutations associated with an HCM phenotype were described before the introduction of next generation sequencing (NGS) by the screening of the Z-line, the cardiac-specific N2B (exon 49) and the N2A domain (exon 103 and 104) (Itoh-Satoh *et al.;* 2002). In 1999, Satoh and colleagues published a Z-disk missense variant, p.Arg740Leu, in a single patient with HCM and showed increased binding affinity to α-actinin in a yeast 2-hybrid assay (Satoh *et al.;* 1999). A second missense variant, p.Ser4116Tyr, in the N2B exon led to enhanced TTN binding to the LIM protein FHL2 (Itoh-Satoh *et al.;* 2002) (Takahashi *et al.;* 2005). Two other variants in the N2A domain showed abnormal localization of CARP, which is known to interact with the TTN/N2A domain (Arimura *et al.;* 2009).

Recently, more comprehensive sequencing studies using next generation sequencing have shown a very small contribution of *TTN* truncating variants in HCM at a range of 1.3%-4%. Herman and colleagues analyzed 231 patients with hypertrophic cardiomyopathy by NGS, and in 3 patients (1.3%) reported a truncating variant; the finding of truncating variants in the control population was even higher (3%) than in the HCM cohort (Herman *et al.;* 2012). Another study by Lopes and colleagues investigated 41 cardiovascular genes in 223 patients with HCM using NGS (Lopes *et al.;* 2013). They found 219 rare titin variants in 142 patients (63.6%). However, of the 219 individuals, 193 carried a *TTN* variant in addition to a likely disease-causing mutation in another gene. Only 30 patients carried an isolated heterozygous variant, with 10 patients (4% of the cohort) having a variant predicted to cause loss of function. This was more than the proportion reported by Herman and colleagues for HCM but

lower than the proportion found in their own control exome population (UK10K project) (Herman *et al.;* 2012) (Lopes *et al.;* 2013). Recent data demonstrate that the ongoing difficulties in understanding the role of *TTN* variants in hypertrophic cardiomyopahty are more supportive of titin as a possible disease-modulating factor rather than as the primary cause of disease (Gerull *et al.;* 2015). However, recent NGS studies revealed that:

(1) *TTN* variants are most often associated, in the same HCM patient, with mutations in other sarcomeric or HCM-candidate genes (Lopes *et al.;* 2013),

(2) The prevalence of isolated titin mutations predicting loss-of-function in HCM cohorts is between 3% and 4%, which is comparable or even lower than that of the prevalence of *TTN* truncating mutations in the normal healthy control population (Herman *et al.;* 2012) (Lopes *et al.;* 2013).

Titin mutations also appear to cause arrhythmogenic right ventricular dysplasia (ARVD), a disease characterized by right ventricular dysfunction and ventricular arrhythmias (Taylor *et al.;* 2011). There are no functional studies for the *TTN* changes identified in HCM patients, and the informativity of cosegregation analysis in their families is limited. Therefore, the implication of titin mutations in the pathogenesis of hypertrophic cardiomyopathy remain unclear and no genotype–phenotype correlation can be established at present (Chaveau *et al.;* 2014). Finally, mutations of genes encoding proteins that interact with and/or bind to titin are responsible for both DCM and HCM. Until now, it has been shown than pathogenetic *TTN* mutations are common in general population and one hypothesis is that these variants act like a phenotypic modifier concurrently to the presence of sarcomeric mutations in the pathogenesis of hypertrophic cardiomyopathy. Indeed, titin variants with MAF $\leq$ 0,05 have been described in patients with HCM in association with rare sarcomeric mutations (MAF $\leq$ 0,01). Targeting titin as a therapeutic strategy could potentially involve manipulation of isoforms, post-translational modifications, and up-regulation of normal protein in patients with disease causing mutations (LeWinter *et al.;* 2014). The central role of TTN means that different mutations can have significantly diverse biological effects and result in different clinical manifestations.

1.8 PATHOGENESIS OF HYPERTROPHIC CARDIOMYOPATHY

Hypertrophic cardiomyopathy is a very common genetic heart disease present in one in 500 in the general population and can affect people of any age but this ratio is underestimated due

to the lack of information regarding familial cases or asymptomatic subjects (Marques *et al.;* 2016) (Mozaffarian *et al.;* 2016). The clinical profile of HCM is quite heterogeneous. While some patients exhibit severe to mild manifestations, others don't know that they have the disease (Marques *et al.;* 2016). The initial suspicious of HCM come from a disomogeneousheartbeat during physical activity, family history, or an abnormal echocardiogram (ECG) pattern (Marian, 2010) (Maron *et al.;* 2012) (Maron *et al.;* 2013) (Marques *et al.;* 2016). Its diagnosis is based on two-dimensional echocardiography, which permits the detection of an asymmetric hypertrophied left ventricle chamber. Other HCM clinical manifestations include left ventricular hyper-contractility, cardiac insufficiency, ventricular fibrillation, syncope and arrhythmias. Regarding its morphological and histological features, left ventricle wall and ventricular septum thickening typically occurs (Teare, 1958) (Maron *et al.;* 1979) (Varnavaer *et al.;* 2001) (Marques *et al.;* 2016). The architecture of the hypertrophic myocardial fibers differs in shape and angle arrangement, leading to a chaotic environment (Maron *et al.;* 1981). In combination with cellular disarray, fibrosis with an abnormal collagen matrix is also observed (St. John Sutton *et al.;* 1980) (Shirani *et al.;* 2000) (Kwon *et al.;* 2009) (Marques *et al.;* 2016) (Nakamura *et al.;* 2016). Hypertrophic cardiomyopathy demonstrates age-dependent penetrance, affecting 50–80% and 95% of individuals by age 30 and ages 50–60, respectively. Survival to 75 years or beyond has been estimated in approximately 25% of an unselected HCM cohort (Force *et al.;* 2010). More than 1500 mutations have been described in association this disease: pathogenic mutations are found in 50–60% of familial HCM and in 30-40% of apparently sporadic cases with no family history (Richard *et al.;* 2003) (Ho *et al.;* 2015). The 90% of pathogenic mutations altering physical and functional properties of proteins are missense, in which a single normal amino acid is exchanged for another. Missense mutations at codons conserved between species and/or isoforms are more likely to be pathogenic than mutations at poorly conserved regions. Alternatively, more radical mutations affect many amino acids in the protein, resulting in a very different product (i.e., frameshift), and are generally predicted to carry more substantial clinical consequences. Frameshift mutations are caused by insertion or deletion of ±1 nucleic acids in the coding region often resulting in shortened truncated proteins (frequently found in the *MYBPC3* gene), or abnormal splicing of messenger ribonucleic acid (mRNA) (Maron *et al.;* 2012). Some genes, or some particular mutations, were described as associated with a high degree of sudden death; more specifically the mutation p.Arg403Gln that occur in the *MYH7* gene is associated with increased risk of heart failure and sudden death (Watkins *etal.;* 1995) (Force *et al.;* el 2010) (Ho *et al.;* 2015).

Mutation in *TNNT2* can cause HCM with little hypertrophy, or even normal cardiac morphology, both in transgenic animals (Frey *et al.;* 2000) and in humans (Watkins *et al.;* 1995). Individuals carrying these mutations may have a high risk of malignant ventricular arrhythmias and sudden cardiac death (Watkins *et al.;* 1995) (Frey *et al.;* 2000). Mutations in *MYBPC3* have been associated with incomplete penetrance, late-onset and benign course of the disease (Charron *et al.;* 1998 Erdmann *et al.;* 2001). By contrast, in another study, mutations in *MYBPC3* have been associated with a more severe prognosis (Oliva-Sandoval *et al.;* 2010), underlining, again, as the genotype-phenotype correlations in HCM are quite variable. Similar to other sarcomeric genes, *TPM1* gene mutations are also correlated with hypertrophic and dilated cardiomyopathy phenotypes (Thierfelder *et al.;* 1994) (Olson *et al.;* 2001) but occur at very low frequencies (around less than 1%), as reported by large-scale studies (Richard *et al.;* 2003) (Van Driest *et al.;* 2003) (Marques *et al.;* 2016). The incorporation of specific mutants (A63V, K70T, D175N, and E180G) into adult cardiomyocytes revealed different isometric force measurements at submaximal Ca^{2+} concentrations, suggesting that HCM-related mutants would predict clinical severity (Michele *et al*.; 1999). May also occur damage to the electrical signal conduction, leading to arrhythmia, tachycardia and ventricular fibrillation, which may ultimately contribute to the development of secondary pathologies, e.g., ischemia or hypotension (Kon-No *etal.;* 2001) (Christiaans *et al.;* 2009) (Lan *et al.;* 2013) (Crocini *et al.;* 2016) (Marques *et al*.; 2016). Altered ion channels including at least six susceptible genes, e.g., *KVLQT1*, *HERG*, *SCN5A*, *minK*, *MiRP1*, and *RyR2* play critical steps during the development of arrhythmia phenotypes (Keating *et al*.; 2001). Of note, the ryanodine channel (*RyR2*) triggers the release of Ca^{2+} from the sarcoplasmic reticulum to start contraction. Mutations in *RyR2* lead to aberrant intracellular Ca^{2+} metabolism and Ca^{2+} overload that may have an involvement in arrhythmias (Keating *et al.;* 2001). Most HCM patients are heterozygous for the mutation, but in 3-5% of cases, patients carry two mutations in the same gene (compound heterozygous or homozygous) or in different genes (digenic). This is generally associated with a more severe phenotype with younger age of onset (often < 10 years) and more adverse events suggesting a gene-dosage effect (Richard *et al.;* 1999) (Richard *et al.;* 2000) (Richard *et al.;* 2003) (Ho *et al.;* 2000) (Lekanne *et al.;* 2006). Like in other genetic diseases, identified mutations in HCM patients can be pathogenic (disease causing), silent polymorphisms, or unclassified variants of which the pathogenic effect is still unclear. Indeed, distinguishing pathogenic mutations from VUS (variants of uncertain significance), or rare non-pathogenic variants, has increasingly emerged as a dilemma for interpreting testing results in HCM

(Tester *et al.;* 2011) (Maron *et al.;* 2012). In general, the guidelines for interpreting VUS are currently absent, and this type of variants have frequencies estimated from 5% to 50%, largely dependent on the number of pathogenicity classes used to categorize mutations and the number of genes in the testing panel. From a human genetics perspective, there is still more to learn about hypertrophic cardiomyopathy. It is clear that HCM is a truly complex disease that can present at any age with variable hypertrophy and outflow tract obstruction, and it can advance in an innocuous progression, or predispose individuals to arrhythmia and sudden cardiac death. This clinical heterogeneity is matched by the genotypic heterogeneity associated with the pathogenesis of this disease. While the mutated genes that serve as the molecular substrates for this disease are becoming increasingly understood, the mechanistic link between HCM-susceptibility mutation and disease pathogenesis and expressivity remains a significant challenge to elucidate (Maron *et al.;* 2012). Genetic testing was initially possible only in research laboratories capable of performing linkage analysis and candidate gene sequencing in large, well-characterized families with obvious inherited disease. However, advances in contemporary DNA-sequencing methodology make gene-based diagnosis increasingly feasible in routine clinical practice. Faster and more affordable genetic testing provides a lot of opportunities to improve diagnostic certainty when evaluating patients and families with relatively non-specific phenotypes of cardiac hypertrophy, dilation, and contractile dysfunction (Ho *et al.;* 2015).

1.9 SECONDARY CARDIOMYOPATHIES: PHENOCOPIES OF HYPERTROPHIC CARDIOMYOPATHY

Secondary cardiomyopathies show pathological myocardial involvement as part of a large number and variety of systemic multiorgan disorders. HCM is a complex phenotype generally determined by mutations on sarcomeric genes but in about 50% patients with unexplained LVH (left ventricular hypertrophy) no mutation in sarcomeric or sarcomere-related genes was found (van Driest *et al.;* 2005) (Alcalai *et al.;* 2008). Likewise, epigenetic factors, microRNAs and protein modifications such as phosphorylation, acetylation and glycation are expected to contribute to phenotypic expression of cardiac hypertrophy (Marian *et al.;* 2008). The discovery of mutations in these genes provided insights into clinical features that distinguish different histopathology and pathophysiology in cardiac hypertrophy. Hyperthophyc cardiomyopathy could be linked to different diseases such as metabolic syndromeswith pathologic vacuoles containing glycogen or intermediary metabolites (Pompe

disease, Anderson-Fabry disease, PRKAG2/Wolff-Parkinson-White syndrome, LAMP2/Danon disease), mitochondrial diseases, nucleotide repeat syndromes, congenital malformation syndromes (Noonan syndrome, Cardiofaciocutaneous syndrome, Cantù syndrome) and other diseases. Diagnostic distinction between sarcomeric HCM and its phenocopieshas showed the limitations of the clinical diagnosis oh HCM and emphasized the need to discover the important determinants of the cardiac hypertrophic responseto elucidate the pathogenesis of cardiac hypertrophy and deliver effective therapy.

For example, mutations in *LAMP2* are usually associated with rapid and potentially lethal clinical course within the first 3 decades, requiring early consideration for heart transplant (Maron *et al.;* 2009) (Maron *et al.;* 2010) (Maron *et al.;* 2012). In Fabry disease, clinical benefits have been attribuited to enzyme replacement theraphy with recombinant α-galactosidase A, including regression of left ventricular hypertrophy and improbe myocardial function and exercise capacity (Maron *et al.;* 2012). Collectively, these disorders define a subset of unexplained hypertrophy rather than common HCM.

Pompe disease (OMIM 232300): Glycogen storage disease type II, commonly reported as Pompe disease, is a rare autosomal recessive disorder, caused by a deficiency of the lysosomal acid α-glucosidase (acid maltase, GAA) enzyme, resulting in a massive lysosomal glycogen accumulation in cardiac and skeletal muscles. Two forms of this disease are considered: the infantile form, with massive cardiac hypertrophy and muscle weakness characterized by prominent mortality rate during the first year of life due to cardiac and/or respiratory failure, and the late-onset disease form, which is characterized by a progressive course of muscle weakness. The involvement of respiratory muscles ultimately leads to premature death. The difference between these two forms has been shown to originate from the wide variety of mutations on the *GAA* gene and the level of residual enzyme activity. The effectiveness of enzyme replacement therapy (ERT) depends on the ability of cells to internalize synthetically recombinant lysosomal enzymes via the mannose-6-phosphate receptor pathway followed by their delivery to the lysosomes, where the replacement of the defective enzyme with the functional enzyme occurs. Clinical studies the use of a recombinant analog of α-glucosidase, alglucosidasealfa (Myozyme) have shown a significant increase in lifetime in parallel with prominent decrease of cardiac hypertrophy, in both infantile and late-onset forms (Kishnani *et al.;* 2007) (Leviner *et al.;* 2015). In the last years, scientists developed alternative therapeutic strategies for Pompe disease: the combination of ERT with immune-modulatory agents such as methotrexate account for the reduction of

immune response against the recombinant enzyme (Joly *et al.;* 2014) (Leviner *et al.;* 2015). Than, pioneering experiments in GAA-KO mice have shown that the inhibition of glycogen synthase by either RNA interference techniques or by negatively regulating it via its signaling pathway both enhanced the beneficial effect of ERT, due to reduction in lysosomal glycogen storage. Therefore, these techniques might be considered as a novel therapeutic option for Pompe patients (Clayton *et al.;* 2014) (Leviner *et al.;* 2015).

Anderson-Fabry disease (OMIM 301500): The Fabry disease is an X-linked lysosomal storage disorder resulting from a deficiency of the lysosomal enzyme β-galactosidaseA (α-GalA) encoded by *GLA* gene, leading to the accumulation of globotriaosylceramide (Gb3) in various tissues (Nakao *et al.;* 1995) (Leviner *et al.;* 2015) (Seydelmann *et al.;* 2015). Absent or reduced enzyme activity leads to the inability to catabolize globotriaosylceramide (Gb3) and related glycosphingolipids, with a progressive intracellular storage of Gb3 in various tissues and organs and an elevated plasma concentration of lyso-Gb3 (De Francesco *et al.;* 2013) (Seydelmann *et al.;* 2015). The intracellular accumulation of Gb3 also occurs within myocytes, valves and vascular endothelium of the heart (Nakao *et al.;* 1995) (Duro *et al.;* 2014)(Seydelmann *et al.;* 2015). Reported incidence is about 1:40,000 worldwide (Seydelmann *et al.;* 2015), *GLA* is the only gene currently known to be associated with Fabry's disease, spans approximately 13 kb of genomic DNA and contains seven exons, encoding a 429-amino acid polypeptide that includes a 31-amino acid signal peptide. Principal manifestations include peripheral and autonomic neuropathy, nephropathy, premature stroke, and white matter lesions and cardiomyopathy. More than 50% of all Fabry patients have a cardiac involvement (Fabry cardiomyopathy), most frequently concentric left ventricular hypertrophywithout LV outflow tract obstruction (Weidemann *et al.;* 2005) (Barbey *et al.;* 2006) (Linhart *et al.;* 2007) (Seydelmann *et al.;* 2015). Cardiomyopathy usually develops at a relatively advanced stage but constitutes a major cause of mortality. It typically manifests in middle-aged males and elderly females as cardiac hypertrophy with diastolic dysfunction and involvement of the conduction tissue, which eventually leads to conduction block and arrhythmia (Leviner *et al.;* 2015). Furhermore, cardiac manifestations are arrhythmias, chronic heart failure and small vessel disease. Malignant arrhythmias are the predominant cause for the substantially increased morbidity and reduced life expectancy (Mehta *et al.;* 2005) (Seydelmann *et al.;* 2015). The ventricular hypertrophy increases with disease progression. This distinguishes Fabry cardiomyopathy from idiopathic hypertrophic cardiomyopathy and can be used to screen Fabry patients among individuals with

unexplained LV hypertrophy (Monserrat *et al.;* 2007) (Seydelmann *et al.;* 2015). The end-stage Fabry cardiomyopathy is characterized by intramural replacement fibrosis also limited to the basal postero-lateral wall of the LV (Moon *et al.;* 2003) (Weidemann *et al.;* 2009) (Seydelmann *et al.;* 2015). The replacement fibrosis seems to be the main cause for cardiac arrhythmias like bradyarrhythmias or malignant ventricular arrhythmias, both leading to a bad prognosis for Fabry patients (Shah *et al.;* 2005) (Weidemann *et al.;* 2005) (Takenaka *et al.;* 2008) (Seydelmann *et al.;* 2015). Heterozygous females typically have milder symptoms at a later age, but phenotypic expression in women may range from nearly asymptomatic throughout life to symptoms as severe as those observed in males with the classical phenotype. The most common instrument to screen for Fabry cardiomyopathy is echocardiography, which is highly available and easily applicable. A cardiologist together with the other involved disciplines should define the indication for enzyme replacement therapy that has been approved since 2001 (Leviner *et al.;* 2015).While substrate manipulation and supporting care includingmedications, pacemaker/defibrillators, dialysis, and pain control are part of the therapeutic arsenal, ERT revolutionized the natural history of this disease, in particular when initiated before the occurrence of irreversible organ damage. Before and during ERT it is important to initiate additional therapy in almost every Fabry patient: novel approaches are developed to potentiate the effect of ERT.A recent study by Lukas and his groupdiscovered two new drugs that might be potential enhancers of pharmacological chaperones. The first is the expectorant ambroxol, especially in combinationwith migalastat, could raise α-Gal A levels in a cell free thermal denaturation test. The second, the PPAR-γagonist-rosiglitazone was also tested in a similar modal and showed promising results, especially in combination with migalastat (Leviner *et al.;* 2015) (Lukas *et al.;* 2015).

PRKAG2/Wolff-Parkinson-White syndrome (OMIM 144200): Molecular studies of patients characterized by hypertrophic cardiomyopathy but without sarcomere-protein gene defects have led to the identification of other genetic causes of cardiac hypertrophy, including mutations in *PRKAG2* (protein kinase AMP-activated non-catalytic subunit gamma 2)(Gollob *et al.;* 2001) (Arad *et al.;* 2002a) (Arad *et al.;* 2005).

PRKAG2 encodes for γ2 subunit of AMPK (AMP activated protein kinase), a highly conserved heterotrimeric enzyme that is expressed in most mammalian tissues, including heart muscle. In humans *PRKAG2* missense mutations cause glycogen-storage cardiomyopathy: studies both humans and animal models have demonstrated that these

mutations in the γ subunit lead to constitutive activation of PRKAG2. The alteration of PRKAG2 leads to altered glycogen metabolism and to dramatic accumulation of cardiac glycogen (Arad *et al.;* 2005). *PRKAG2* mutations cause myocite hypertrophy by stimulating glycogen-filled vacuoles but cause neither myocite disarray nor interstitial fibrosis, which typically happen with defects of sarcomere-protein genes (Yeh *et al.;* 1980) (Sim *et al.;* 1988) (Arad *et al.;* 2007).

Whether activated AMPL also triggers other pathways that many contribute to the increase in cardiac mass or electrophysiological abnormalities is unknown (Arad *et al.;* 2005). These biochemical studies suggest mechanism by which *PRAKG2* missense mutations determine AMPK activity. Under normal physiological condition, the heart has abundant ATP and nearly absent AMP. Under these conditions, high ATP levels inhibit AMPK. AMPK carryng *PRKAG2* mutations has decreased affinity for ATP, and increasing baseline AMPKactivity. AMPK appears to have a role in regulating Akt1-indiced hypertrophy in neonatal cardiomyocitesbut the role of AMPL in cardiac hypertrophy remains unknown (Chan *et al.;* 2004) (Arad *et al.;* 2007)

Danon disease (DD, OMIM 300257): It is a rare monogenic metabolic X-linked disorder characterized by early-onset cardiomyopathy with hypertrophic or dilated phenotype (frequently responsible for fatal outcome), intellectual disability, and proximal myopathy (Danon *et al.;* 1981) (Bottillo *et al.;* 2016c). The exact prevalence of DD is unknown; however, it has been reported in 1-6% of patients with unexplained left ventricle hypertrophy (Charron *et al.;* 2004) (Arad *et al.;* 2005) (Yang *et al.;* 2005) (Hedberg *et al.;* 2015) (Bottillo *et al.;* 2016c) and in up to 17% of patients with LVH and other features such as elevated serum creatine kinase (CK) or Wolff-Parkinson-White (WPW) syndrome (Arad *et al.;* 2005) (Hedberg *et al.;* 2015) (Bottillo *et al.;* 2016c).

DD is caused by LAMP2 (lysosome-associated membrane protein 2) defects which coats the inner surface of the lysosomal membrane and is supposed to act as a receptor for proteins to be imported and degraded within lysosomes in chaperone-mediated autophagy (Eskelinen *et al.;* 2002) (Bottillo *et al.;* 2016c).

The lysosomes fail to fuse with autophagosomes and the cell is left with undegraded contents such as protein aggregates, malfunctional organelles, and old glycogen. For this reason, the disease was originally named Pompe with normal acid maltase (Leviner *et al.;* 2015). Danon disease is an example of primary failure of autophagy. It is now estimates that secondary impairment of autophagy also contributes to the pathophysiology of other lysosomal storage

diseases by the accumulation of toxic protein aggregates and defective mitochondria (Choi *et al.;* 2013) (Leviner *et al.;* 2015).

*LAMP2*is located on chromosome Xq24, and its open reading frame consists of 1233 nucleotides spanning nine exons and encoding 410 amino acids (Nishino *et al.;* 2000) (Bottillo *et al.;* 2016c). Exon 9 undergoes alternative splicing generating three splice isoforms (LAMP2A, LAMP2B, and LAMP2C) that differ in the transmembrane and cytoplasmic domains but have identical luminal domains (Eskelinen *et al.;* 2002) (Bottillo *et al.;* 2016c). LAMP2B isoform is mainly expressed in heart, skeletal muscle, and brain (the three "target tissues" of DD) (Di Mauro *et al.;* 2007) (Bottillo *et al.;* 2016c) while isoforms LAMP2A and LAMP2C are nearly ubiquitous (Konecki *et al.;* 2005) (Bottillo *et al.;* 2016c). To date, more than 100 genetically proven cases of DD have been reported, and most of the mutations are small deletions/insertions, nonsense, or splicing alterations (Cheng *et al.;* 2012) (Majer *et al.;* 2014) (Bottillo *et al.;* 2016c).

Over several years, the heart progresses to the hypokinetic stage and develops severe heart failure leading to death unless heart transplantation takes place (Leviner *et al.;* 2015). Clinical manifestations are variable but normally more severe in males due to the X-linked dominance (Boucek *et al.;* 2011) (Bottillo *et al.;* 2016c). Typically, a teenage boy has rapidly progressive hypertrophy, which may reach big dimensions and be associated with Wolff-Parkinson-White syndrome and arrhythmia (Arad *et al.;* 2005) (Leviner *et al.;* 2015). However, early-onset cases with unfavorable prognosis have been rarely described in females (Yang *et al.;* 2005) (Maron *et al.;* 2009) (Kim *et al.;* 2010) (Dara *et al.;* 2011) (Miani *et al.;* 2012) (Hedberg *et al.;* 2015) (Bottillo *et al.;* 2016c). Male patients also have transaminase elevation without clinically significant liver disease (Arad *et al.;* 2005). These are believed to be caused mainly by an unfavorable skewing of X-chromosome inactivation (XCI) in the cardiac tissue (Fanin *et al.;* 2006) (Majer *et al.;* 2014) (Hedberg *et al.;* 2015) (Bottillo *et al.;* 2016c). Electrophysiological abnormalities follow but may precede the development of cardiomyopathy. Because the stress of pregnancy and labor may lead to heart failure, some cases are wrongly diagnosed as peripartum cardiomyopathy, and correct diagnosis is reached only after the development of a typical disease in a male offspring (D'Souza *etal.;* 2014) (Leviner *et al.;* 2015). A lot of knowledge needs to be learned about the process of autophagy, the modification of the processes triggering autophagy may help modify the course of this lethal disease, while a targeted protein therapy would facilitate the restoration of the lysosomal function in Danon disease (Leviner *et al.;* 2015).

Mitochondrial diseases: Primary mitochondrial respiratory chain diseases are systemic disorders caused by sporadic or inherited mutations in nuclear or mitochondrial DNA (mtDNA). Their main unifying feature is an alteration in energy homeostasis caused by decreased ATP production (Giordano *et al.;* 2013). The main tissues with high-energy requirements involved in these diseases are brain, skeletal muscle, and heart. The presentation of mitochondrial DNA defects is more variable and age-dependent, related to tissue distribution of defective mitochondria, mutation load, and mitochondrial aging (Holmgren *et al.;* 2003) (Scaglia *et al.;* 2004) (Leviner *et al.;* 2015). Organ dysfunction is caused by energy deficiency, oxygen radical damage, and proapoptotic signaling by the damaged mitochondria.

According with the literature, cardiac involvement is reported in about 20% to 25% of patients with mitochondrial disorders (Holmgren *et al.;* 2003) (Scaglia *et al.;* 2004) (Limongelli *et al.;* 2010) (Giordano *et al.;* 2013). Cardiac manifestations include cardiomyopathies that variably affect the patients' clinical outcome (Berardo *et al.;* 2011) (Giordano *et al.;* 2013). The most frequently reported phenotype is hypertrophic cardiomyopathy often caused by mutations in nuclear genes that affect the activities of the complexes of the mitochondrial respiratory chain (Antonicka *et al.;* 2003) (Haack *et al.;* 2010) (Gotz *et al.;* 2011) (Ghezzi *et al.;* 2012) (Giordano *et al.;* 2013). However, a substantial proportion of cases remain genetically undiagnosed. This is the case of mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes (MELAS, OMIM 540000) (Limongelli *et al.;* 2010) (Haack *et al.;* 2010) (Berardo *et al.;* 2011) (Gotz *et al.;* 2011) (Bated *et al.;* 2012) (Ghezzi *et al.;* 2012) (Hollingsworth *et al.;* 2012) (Giordano *et al.;* 2013), caused by heteroplasmic mutations (coexistence of wild-type and mutated mtDNA molecules in the same cell) in the mitochondrial gene *MTTL1*, coding for leucine (UUR) tRNA. A recent study conducted by Hollingsworthand colleagues, showed that cardiac hypertrophy is a constant feature in patients with this point mutation m.3243ANG in *MTTL1*gene (Hollingsworth *et al.;* 2012).

Furthermore, Taylor and colleagues described two families with HCM associated with a homoplasmic A4300G mutation in the mt-tRNA^{Ile}gene. In both families, the clinical traits and associated histochemical and biochemical abnormatilies were confined to the heart. This group studied the cardiac tissue of affected individuals which revealed not only severely decreased respiratory chain activity but also very low steady state levels of mt-tRNA^{Ile} (Taylor *et al.;* 2003). The mutation affects a base pair in the secondary structure of mt-tRNA^{Ile} in a region of the gene that shows high evolutionary conservation. There was a

mildly asymmetric pattern of LV hypertrophy, with some patients showing a prominent thickening of the posterior wall and others of the ventricular septum (Taylor *et al.;* 2003).

An othermithocondrial disease often associated with cardiac hypertrophy is Kearns–Sayre syndrome (KSS, OMIM 530000). The typical phenotype of KSS includes progressive external ophthalmoplegia, pigmentary retinopathy, cardiac conduction defects and less commonly cardiac hypertrophy (Ashizawa *et al.;* 2001) (Marian *et al.;* 2008). L-Carnitine deficiency, caused by mutations in chromosomal genes encoding solute carrier family 22, member 5 (*SLC22A5*) or OCTN2 transporter, could manifest as either concentric or eccentric cardiac hypertrophy (Guertl *et al.;* 2000) (Marian *et al.;* 2008). Similarly, mutations in mitochondrial carnitine palmitoyltransferaseI (*CATI*) and translocase (*SLC25A20*) are also associated with cardiac hypertrophy and heart failure (Kelly *et al.;* 1994) (Guertl *et al.;* 2000) (Marian *et al.;* 2008).

In general, a muscle biopsy may be useful to confirm the clinical suspicion, although a mitochondrial etiology cannot be excluded based on negative histology, the natural history of isolated mitochondrial cardiomyopathies is often characterized by a rapid progression to congestive heart failure, especially in male patients (Taylor *et al.;* 2003) (Perli *et al.;* 2012) (Giordano *et al.;* 2013).

Nucleotide repeat syndromes: Cardiac involvement is a major determinant of morbidity and mortality in triplet repeat syndromes (Korade-Mirnics *et al.;* 1998) (Cummings *et al.;* 2000) (Marian *et al.;* 2008). An example of hypertrophic cardiomyopathy phenocopiesis this class of neuromuscular disorders caused by the expansion of the trinucleotide repeats in various genes, such asmyotonic dystrophy type 1 (DM1, OMIM 160900), Huntington's disease (HD, OMIM 143100) and fragile site syndromes (Cumming *et al.;* 2000) (Marian *et al.;* 2008). The common form of myotonic dystrophy is caused by the expansion of GC-rich triplet repeats in the 3'-untranslated region of *DMPK*, which encodes myotonicdystrophy protein kinase. Cardiac hypertrophy, often diagnosed as HCM, and conduction defect are the prominent clinical features due to progressive degeneration of muscles and myotonia (Korade-Mirnics *et al.;* 1998) (Marian *et al.;* 2008). Cardiac hypertrophy or dilatation also occurs in patients with Friedreich's ataxia (FRDA, OMIM 229300), which is caused by the expansion of GAA repeat sequences in the *FRDA* gene (Palau, 2001) (Marian *et al.;* 2008). The severity of clinical manifestations of triplet repeat syndromes correlates with the size of the repeats (Bit-Avragim *et al.;* 2001) (Marian *et al.;* 2008).

FHL1-related diseases: Severe left ventricular diastolic dysfunction (LVDD) in individuals with hypertrophic cardiomyopathy has been associated with poor exercise tolerance and prognosis in both adults and children with the disease (Matsumura *et al.;* 2002) (McMahon*et al.;* 2004) (Harris *et al.;* 2006) (Efthimiadis *et al.;* 2007) (Kubo *et al.;* 2007) (Biagini *et al.;* 2009) (Maskatia *et al.;* 2012) (Hartmannova *et al.;* 2013). Severe LVDD may occur as an initial manifestation of HCM with limited LV hypertrophy, or it may represent advanced disease, an alternative to end-stage dilated HCM (Harris *et al.;* 2006) (Hartmannova *et al.;* 2013). Recently, Hartmannova and colleagues report a novel mutation of *FHL1* c.647_648 insTencoding four-and-a-half LIM domain protein 1 (FHL1) in 3 hemizygous males on a large family with isolated X-linked HCM with severe LVDD. Mild cardiac involvement in heterozygous females included asymptomatic abnormalities of ECGF with a modest increase in LV mass since the sixth decennium and 1 case of apical HCM in ninth decennium. More than 30 mutations of *FHL1* have been associated with 5 different X-linked myopathies: reducing body myopathy (OMIM 300718), scapuloperoneal myopathy (OMIM 300695), X-linked myopathy with postural muscle atrophy (OMIM 300696), rigid spine syndrome, and Emery-Dreifuss muscular distrophy (OMIM 310300) with a variable cardiac involvement presenting as dilated or HCM (Windpassinger*et al.;* 2008) (Gueneau *et al.;* 2009) (Knoblauch*et al.;* 2010) (Shathasivam*et al.;* 2010) (Cowling *et al.;* 2011) (Hartmannova *et al.;* 2013)

Noonan syndrome (NS, OMIM 163950): Noonan syndrome is probably the most common cause of HCM phenocopy in children. It is an autosomal dominant condition with a variable phenotype comprising short stature, congenital heart defects and minor facial anomalies (Noonan, 1968) (Jongmans *et al.;* 2005). Its prevalence has not been determined accurately to date, most autohors cite thefigure of 1 in 1000-2500 live births but that estimate is not based on a population study (Nora *et al.;* 1974) (Tartaglia *et al.;* 2010). NS is a Mendelian trait and, typical for that genetic mechanism, 1/3 to 1/2 of cases arise from spontaneous mutations (Tartaglia *et al.;* 2010). Thus far, seven genes (*PTPN11*, *SOS1*, *KRAS*, *NRAS*, *RAF1*, *BRAF*, *MEK1* and *RIT1*) have been causally related to this disorder (Tartaglia *et al.;* 2010) (Aoki *et al.;* 2013).

The main facial findings of NS are hypertelorism with down-slanting palpebral fissures, ptosis, and low-set posteriorly angulated ears with a thickened helix. The cardiovascular defects most commonly associated with this condition are pulmonary stenosis and hypertrophic cardiomyopathy. Other manifestations are webbed neck, chest deformity, mild

mental retardation, cryptorchidism, feeding difficulties, bleeding diathesis, and lymphatic dysplasia (Jongmans *et al.;* 2005).

Cardiofaciocutaneous syndrome (CFCS, OMIM 115150): It is a rare, sporadic, multiple congenital anomalies/mental retardation syndrome characterized by failure to thrive, severe feeding problems, developmental delay, reduced growth, distinctive dysmorphic facial features, abnormalities of the skin, gastrointestinal tract and central nervous system, and cardiac defects (Reynolds *et al.;* 1986) (Roberts *et al.;* 2006) (Tartaglia *et al.;* 2010). CFCS is genetically heterogeneous, with mutations in the *KRAS*, *BRAF*, *MEK1* and *MEK2* genes occurring in approximately 60-90% of affected individuals (Narumi *et al.;* 2007) (Niihori *et al.;* 2006) (Rodriguez-Viciana *et al.;* 2006) (Nava *et al.;* 2007) (Schulz *et al.;* 2008) (Tartaglia *et al.;* 2010). CFCS has considerable clinical overlap with NS, and "borderline" cases are commonly observed, which justified the long debated question of whether it was a separate nosologic entity or an extreme phenotype/variant of NS (Wieczorek *etal.;* 1997) (Fryer *et al.;* 1991) (Neri *et al.;* 1991) (Tartaglia *et al.;* 2010).
Recurrent facial/head features include relative/absolute macrocephaly, which is usually associated with high forehead, bitemporal narrowing and facial dysmorphia that is coarser compared to NS. The cutaneous involvement includes dry and hyperkeratotic skin (face, arms and legs), ichthyosis, eczema, sparse, friable and curly hair, absent/sparse eyebrows and eyelashes, pigmentary changes (such as café-au-lait spots, naevi or lentigines) and hemangiomata. Heart defects occur in the majority of affected individuals and consist of pulmonic stenosis most commonly, HCM and septal defects (Roberts *et al.;* 2006) (Tartaglia *et al.;* 2010).

Costello syndrome (CS, OMIM 218040): This disorder was originally described in 1971 and delineated for the second time in 1977 by the same author. It is characterized by prenatal overgrowth followed by postnatal feeding difficulties and severe failure to thrive, distinctive coarse facial features, mental retardation, short stature, cardiac defects (most commonly hypertrophic cardiomyopathy, septal defects, valve thickening and/or dysplasia, and arrhythmias), skin and musculoskeletal abnormalities (Costello, 1971) (Costello, 1977) (Hennekam, 2003) (Zampino *et al.;* 2007). By using a candidate gene approach, Aoki and colleagues showed that germline heterozygous missense mutations in the v-Ha-ras Harvey rat sarcoma viral oncogene homolog (*HRAS*) protooncogene cause this syndrome. According to the available data, activating HRAS mutations have been identified in approximately 85% of

subjects with a clinical diagnosis of CS (Aoki *et al.,* 2005)(Estep *et al.;* 2006)(Gripp *et al.;* 2006)(Kerr *et al.;* 2006) (Zampino *et al.;* 2007). It is an autosomal dominant disorders and due to the severely reduced fitness associated with the disorder, CS almost invariably arises sporadically from a de novo mutation. Advanced paternal age and increased paternal–maternal age difference has been documented in CS, suggesting that paternal germline mutations are the origin of disease (Lurie, 1994).

The diagnosis of CS can be difficult and uncertain in the neonatal and infancy periods due to phenotypic overlap with cardiofaciocutaneous syndrome and Noonan syndrome.

Cantù syndrome (OMIM 239850): Cantú syndrome is a rare condition characterized by congenital hypertrichosis, neonatal macrosomia, a distinct osteochondrodysplasia, and cardiomegaly. Cantù et al described a brother and sister and 2 sporadic cases with a syndrome consisting of generalized congenital hypertrichosis, macrosomy at birth, narrow thorax, cardiomegaly, wide ribs, platyspondyly, hypoplastic ischiopubic branches, small obturator foramen, bilateral coxavalga, enlarged medullary canal, Erlenmeyer-flask-like long bones, and generalized osteopenia (Cantù *et al.;* 1982). Notably, cardiac manifestations such as patent ductus arteriosus, ventricular hypertrophy, pulmonary hypertension, and pericardial effusions are present in ~80% of cases (Lazalde *et al.;* 2000) (Grange *et al.;* 2006) (Scurr *et al.;* 2011) (Van Bon *et al.;* 2012). Autosomal recessive inheritance of Cantu syndrome had been suggested but Lazalde and colleagues reported a Mexican family affected by Cantù syndrome with autosomal recessive inheritance.

Van Bon et al identified heterozygosity for 4 different missense mutations in the *ABCC9* gene and they proposed that this syndrome be added to the list of potassium channelopathies with potential therapeutic options. The protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transportersand encodes the channel regulator SUR2, which contains TMD1 and TMD2, an N-terminal domain (TMD0), and two nucleotide-binding folds (NBF1 and NBF2), comprising Walker A and Walker B nucleotide-binding motifs and other conserved sequences (Higgins *et al.;* 2001) (Nichols *et al.;* 2006) (Van Bon *et al.;* 2012). Alternative RNA splicing of the terminal exon of *ABCC9* produces two SUR2 isoforms: SUR2A, predominantly expressed in cardiac and skeletal muscle cells, and SUR2B insmooth muscle (Brian *et al.;* 2007) (Van Bon *et al.;* 2012). The protein SUR2 is located in a cluster of genes on chromosome 12p12.1 with KCNJ8, suggesting co-regulation at the gene level (Flagg *et al.;* 2010) (Van Bon *et al.;* 2012). A similar situation is observed for KCNJ11 and SUR1, which are also located in a cluster of geneson chromosome

11p15.1. The chromosome 12 cluster-genes encoded KATP channel functions primarily in heart, skeletal, and smooth muscle, the chromosome 11 cluster-genes encoded KATP channel shows a predominant role in the neuroendocrine system, and mutations in these genes may lead to hyperinsulinemic hypoglycemia and neonatal diabetes (van Bon *et al.;* 2012). KATP channels is responsible for the intracellular changes in the ADP/ATP ratio, thereby linking the metabolic state of the cell to its membrane potential (Nichols *et al.;* 2006) (Bryan *et al.;* 2007) (de Wet *et al.;* 2010) (Van Bon *et al.;* 2012).

Absence of sarcomeric KATP channels significantly impairs the myocyte's ability to properly distribute Ca^{2+}, decreasing sensitivity to sympathetic nerve signals and predisposingthe patients to arrhythmia and sudden death (Zingman *et al.;* 2002).

2. AIM OF THESTUDY

Hypertrophic cardiomyopathy is an inherited disease of the heart muscle characterized by a relevant heterogeneity of both morphological and clinical features. Considering the extreme genetic heterogeneity of the disease and the cost of genetic testing, several attempts were made to identify the clinical predictors of an underlying mutation. For this reason, despite the growing knowledge on its genetic basis, the establishment of a more precise genotype-phenotype correlation has been difficult to achieve.

The born of next generation sequencing technology has greatly accelerated the study of the molecular basis of genetic diseases, including cardiomyopathies, with important consequences both in clinical practice and scientific research. To date, the target sequencing (TS) is the most widely used approach in genetic and clinical tests, which has already been applied for the diagnosis of hereditary cardiovascular conditions as well as of other diseases may represent a suitable tool. Targeted gene panels were shown to generate results with analytical quality identical to Sanger sequencing, and to have the advantage of being faster and cheaper with better coverage and sensitivity than that used in more expanded analyses. However, the emergence of next generation sequencing platforms imposes increasing demands on statistical methods and bioinformatic tools for the analysis and the management of the huge amounts of data generated by these technologies.

The main purpose of the present study was to develop an informative genetic testing for patients affected by hypertrophic cardiomyopathy. For this reasons 44 italian HCM-patients were selected from San Camillo-Forlanini Hospital (Rome) and they were analyzed by two custom NGS panels: the first panel was designed to analyze coding, intronic junctions and UTR sequences of 62 sarcomeric and non-sarcomeric genes related to cardiomyopathies; the second panel was developed to analyze coding, intronic junctions and UTR sequences of Titin gene, a locus that could act as a genetic modifier in HCM.

The second aim of this study was the analysis of genotype-phenotype correlations in HCM; these results could be a starting point for delineating the contribution of each analysed gene in the onset and clinical variability of HCM.

Finally, the third purpose of this study was the investigation of patients affected by HCM-related diseases, by molecular deepening and an exhaustive literature review.

3. MATERIALS AND METHODS

3.1 PATIENT

Forty-four adults and unrelated patients were selected from those attending an outpatient hospital service dedicated to the diagnosis and management of HCM. Three patients displayed HCM associated with a syndromic phenotype: case 10, 24 and 27 were respectively affected by a mild form of Fabry's disease, by Cantù's syndrome and by Danon's disease. All patients underwent clinical history registration, physical examination, electrocardiography, echocardiography, cardiopulmonary exercise test coupled with ambulatory ECG monitoring. Thirty-two out of 44 patients were evaluated by cardiac magnetic resonance (CMRI). Diagnostic criteria for HCM was defined in adults by a maximal left ventricular wall thickness ≥13 mm on echocardiography, in the absence of other loading conditions (Klues *et al.;* 1995). Family history of sudden cardiac death, syncope episodes and the presence of Non-Sustained Ventricular Tachycardia (NSTV) were defined as described by O'Mahony et al. (2014). Electrocardiographic changes that were considered of clinical significance included abnormal Q waves (0.04 s or 25% depth of an R-wave), LVH (voltage criteria), and marked repolarization changes (e.g. T-wave inversion in at least 2 leads). The QT interval corrected for heart rate was calculated using the Bazett's formula (QTc (ms) = QT / - RR, where RR is the RR interval measured in seconds). Familial HCM cases were defined if at least one additional affected family member with HCM, or one case of sudden cardiac death was present in the pedigree. From every patients, genomic DNA was extracted from peripheral blood by Maxwell® 16 Blood DNA Purification Kit (Promega). All patients gave informed consent for the DNA analyses, which was approved by local ethic committees in accordance with the principles of the Declaration of Helsinki. Table 11 summarizes the clinical features of the patients at evaluation.

3.2. NEXT GENERATION SEQUENCING ANALYSIS

The inherited forms of cardiomyopathies can be caused by mutations in at least many different genes. A literature review about sarcomeric and non-sarcomeric genes correlated to cardiomyopathies was carried out using public database such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/)

and GeneReviews® (https://www.ncbi.nlm.nih.gov/books/NBK1116/). We analyzed 63 sarcomeric and non-sarcomeric genes in order to identify sarcomeric genes mutations in patients affected by HCM and to discover the role of not only sarcomeric loci, but also of many different genes in the onset of hypertrophic cardiomyopathy.

Genomic DNA from peripheral blood was tested by next generation sequencing with two custom designs for the cardiomyopathy panel, based on AmpliSeq strategy (ThermoFisher, Carlsbad, CA, USA). The first panel was designed to analyze coding, intronic junctions and UTR sequences of 62 genes (Fig. 22). Those comprised 12 sarcomeric loci (*ACTC1, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYOM1, NEBL, TNNC1, TNNI3, TNNT2, TPM1*), 9 cytoskeletal (*CRYAB, DES, DMD, DTNA, EMD, FXN, LAMA4, PDLIM3, SGCD*), 9 Z-disk (*ACTN2, ANKRD1, CSRP3, LDB3, MYOZ2, MYPN, NEXN, TCAP, VCL*), 6 desmosomal (*DSC2, DSG2, DSP, FHL2, JUP, PKP2*), 5 intracellular Ca++ homeostasis (*CALR3, CASQ2, JPH2, PLN, RYR2*), 3 genes encoding for K+ and Na+ channels and interacting proteins (*ABCC9, CAV3, SCN5A*) and 18 other non-sarcomeric loci (*BAG3, CTF1, EYA4, GATAD1, GLA, ILK, LAMP2, LMNA, MYLK2, PRKAG2, PTPN11, RAF1, RBM20, TAZ, TMEM43, TMPO, TTR, TXNRD2*).

In summary, the panel included 36 genes known to be associated with HCM and dilated cardiomyopathy (*ACTC1, ACTN2, ANKRD1, BAG3, CALR3, CAV3, CRYAB, CSRP3, DES, EYA4, GATAD1, ILK, JPH2, LAMA4, LDB3, LMNA, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOZ2, MYPN, NEXN, PDLIM3, PLN, RBM20, SGCD, TCAP, TMPO, TNNC1, TNNI3, TNNT2, TPM1* and *VCL*), 7 genes related with arrhythmogenic right ventricular cardiomyopathy and left ventricular non-compaction (*DSC2, DSG2, DSP, DTNA, JUP, PKP2* and *TMEM43*), 7 genes associated to malformation syndromes and storage disorders (*ABCC9, GLA, LAMP2, PTPN11, PRKAG2, RAF1* and *TTR*), 4 genes related to myopathies and neuromuscular disorders (*DMD, EMD, FXN* and TAZ), 3 genes implicated in arrhythmia syndromes/ion-channel disease (*CASQ2, RYR2* and *SCN5A*) and other 5 cardiovascular candidate genes (*CTF1, FHL2, MYOM1, NEBL* and *TXNRD2).*The second panel was designed to analyze coding, intronic junctions and UTR sequences of *TTN* genes. Table 4 summarizes the genes included in the study as well as their function, associated disorder(s), chromosomal position and sequencing details. The design allowed the targeted resequencing of 2890 amplicons (global size: 345,39 kb/patient) by Ion Torrent PGM^{TM} instrument (ThermoFisher, Carlsbad, CA, USA). In order to validate the applied NGS protocol the entire coding sequences of *MYH7, MYBPC3, TNNT2* and *TNNI3* genes of 23/44 patients were at first analyzed by Sanger sequencing.

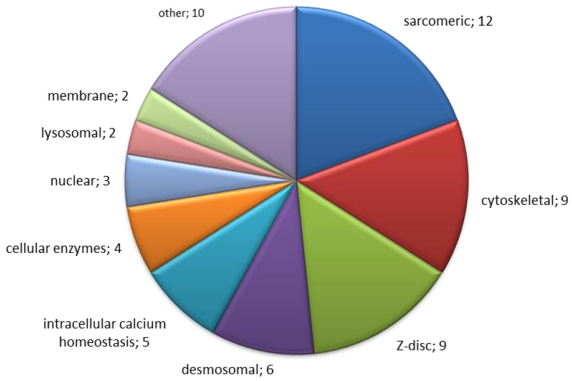


Figure 22. Schematic representation of of the 62 genes included in the first custom NGS panel

No	Gene ID	Description	Cellular Function	Disease symbol	Chromosomal Location	Target (bp)	Amplicons	Coverage (%)	Missed (bp)	Exonic missed (bp)
1	*ABCC9*	ATP-binding cassette, sub-family c	ATP-binding cassette transporter, ATP-sensitive potassium channels	CANTU' SYNDROME;DCM10;ATFB12	chr12:21,950,305-22,089,648	10.022	83	81,4	1.868	178
2	*ACTC1*	Actin, alpha, cardiac muscle 1	Sarcomere, thin filament, muscle contraction	ASD5;DCM1R;HCM11;LVNC4	chr15:35,080,278-35,087,947	3.973	31	97,5	101	0
3	*ACTN2*	Actinin, alpha 2	Actin-binding protein, localized to the Z-disc	DCM1AA	chr1:236,849,751-236,927,578	5.368	46	97,7	126	0
4	*ANKRD1*	Ankyrin repeat domain-containing protein 1	Nuclear transcription factor that negatively regulates the expression of cardiac genes	DCM	chr10:92,671,838-92,681,052	2.334	22	96,4	84	0
5	*BAG3*	Bcl2-associated athanogene 3	Anti-apoptotic activity	DCM1HH;MFM6	chr10:121,410,863-121,437,349	2.729	20	97,7	64	40
6	*CALR3*	Calreticulin 3	Ca(2+)-binding chaperones localized in the endoplasmic/sarcoplasmic reticulum	HCM19	chr19:16,589,849-16,607,023	1.652	15	96,9	52	8
7	*CASQ2*	Calsequestrin 2	Calcium ion reservoir within the sarcoplasmic reticulum of cardiac myocytes	CPVT2	chr1:116,242,607-116,311,446	3.140	28	98,2	55	35
8	*CAV3*	Caveolin 3	Scaffolding protein within caveolar membranes	RMD;MPDT;LQT9;CREATINE PHOSPHOKINASE ELEVATED SERUM;HCM1	chr3:8,775,467-8,788,471	2.735	12	100	0	0
9	*CRYAB*	Crystallin, alpha b (basic)	Cytoskeletal	MFM;DCM1II;CTRCT16;MFM2	chr11:111,779,331-111,782,493	811	7	100	0	0
10	*CSRP3*	Cysteine and glycine-rich protein 3 (cardiac LIM protein)	Positive regulator of myogenesis, role in the organization of cytosolic structures in cardiomyocytes	HCM12;DCM1M	chr11:19,203,559-19,223,609	1.651	16	100	0	0
11	*CTF1*	Cardiotrophin 1	Secreted cytokine that induces cardiac myocyte hypertrophy	-	chr16:30,907,909-30,914,901	1.940	10	62,3	731	369
12	*DES*	Desmin	Muscle-specific class III intermediate filament	LGMD2R;DCM1I;MFM1;SCPNK	chr2:220,283,080-220,291,481	2.608	26	100	0	0
13	*DMD*	Dystrophin	Part of the dystrophin-glycoprotein complex , which bridges the inner cytoskeleton (F-actin) and the extra-cellular matrix; ligand for dystroglycan	DCM3B;DMD;BMD	chrX:31,137,326-33,357,746	19.739	188	98,5	302	
14	*DSC2*	Desmocollin 2	Component of intercellular desmosome junctions	ARVC11	chr18:28,645,923-28,682,408	5.925	52	86,9	777	222
15	*DSG2*	Desmoglein 2	Component of intercellular desmosome junctions	DCM1BB;ARVC10	chr18:29,078,008-29,128,834	6.253	52	87,8	760	220
16	*DSP*	Desmoplakin	Major high molecular weight protein of desmosomes	ARVC8;PPKS2;EPIDERMOLYSIS BULLOSA, LETHAL ACANTHOLYTIC;DCWHK	chr6:7,541,851-7,586,966	11.228	91	98,6	160	80
17	*DTNA*	Dystrobrevin, alpha	Component of the dystrophin-associated protein complex	LVNC1	chr18:32,073,235-32,471,828	16.922	98	93,7	1.074	
18	*EMD*	Emerin	Serine-rich nuclear membrane protein; membrane anchorage to cytoskeleton	EDMD	chrX:153,607,578-153,609,903	1.579	15	100	0	0
19	*EYA4*	Eyes absent homolog 4	Tyrosine phosphatase that promotes efficient DNA repair	DCM1J;DAD10	chr6:133,562,476-133,853,278	6.634	62	98,5	99	7
20	*FHL2*	Four and a half LIM domains 2	Component of intercellular desmosome junctions	-	chr2:105,977,264-106,055,250	2.800	21	97,4	74	
21	*FXN*	Frataxin	Cytoskeletal	FRDA	chr9:71,650,460-71,715,114	7.832	52	81,5	1.452	
22	*GATAD1*	GATA zinc finger domain containing 1	Regulates gene expression	DCM2B	chr7:92,076,746-92,088,762	4.155	24	68	1.330	
23	*GLA*	Galactosidase, alpha	Lysosomal	FABRY disease	chrX:100,652,760-100,663,021	1.698	16	100	0	0
24	*ILK*	Integrin-linked kinase	Receptor kinase regulating integrin-mediated signal transduction; important in the epithelial to mesenchymal transition	DCM1A	chr11:6,624,945-6,632,119	2.916	24	100	0	0
25	*JPH2*	Junctophilin 2	Component of junctional membrane complexes; necessary for proper intracellular Ca(2+) signaling in cardiac myocytes via its involvement in ryanodine receptor-mediated calcium ion release	HCM17	chr20:42,740,318-42,816,238	6.221	49	94,9	317	317
26	*JUP*	Junction plakoglobin	Common junctional plaque protein in desmosomes and intermediate junctions	ARVC12;NAXOS DISEASE	chr17:39,910,840-39,942,984	5.140	37	100	0	0
27	*LAMA4*	Laminin, alpha 4	Noncollagenous constituent of basement membranes	DCM1JJ	chr6:112,429,115-112,575,848	9.735	89	97,5	248	9
28	*LAMP2*	Lysosomal-associated membrane protein 2	Lysosomal	DANON	chrX:119,559,984-119,603,224	12.307	71	90,5	1.169	0
29	*LDB3*	LIM domain binding 3	Cytoskeletal assembly, targeting and clustering of membrane proteins	DCM1C;LVNC3;MFM4	chr10:88,428,187-88,495,844	7.364	59	95	365	0

30	*LMNA*	Lamin A/C	Components of the nuclear lamìna	HEART-HAND SYNDROME, SLOVENIAN TYPE; MALOUF SYNDROME;MUSCULAR DYSTROPHY, CONGENITAL RESTRICTIVE DERMOPATHY,LETHAL;MADA;LGMD1B;FPLD2; HGPS;EDMD3;EDMD2;DCM1A	chr1:156,084,442-156,109,898	3.958	34	95,6	173	0
31	*MYBPC3*	Myosin binding protein c	Myosin-associated protein	DCM1MM;HCM4;LVNC10	chr11:47,352,938-47,374,273	5.577	61	100	0	0
32	*MYH6*	Myosin, heavy chain 6, alpha	Alpha heavy chain subunit of cardiac myosin	ASD3;DCM1EE;HCM14;SSS3	chr14:23,851,180-23,877,506	7.501	73	98,1	142	131
33	*MYH7*	Myosin, heavy chain 7, beta	Heavy chain subunit of cardiac myosin	DCM1S;HCM1;MPD1;LVNC5;MYOPATHY,MYOSIN STORAGE;SPMM	chr14:23,881,928-23,904,890	7.630	77	97,1	225	225
34	*MYL2*	Myosin, light chain 2, regulatory, slow	Regulatory light chain of myosin	HCM10	chr12:111,348,605-111,358,424	1.109	11	100	0	0
35	*MYL3*	Myosin, light chain 3, alkali, slow	Regulatory light chain of myosin	HCM8	chr3:46,899,338-46,904,993	1.209	13	98,1	23	0
36	*MYLK2*	Myosin light chain kinase 2	Myosin light chain kinase; calcium/calmodulin dependent enzyme	HCM1	chr20:30,407,159-30,422,520	3.313	35	99,7	11	0
37	*MYOM1*	Myomesin 1	Major component of the myofibrillar M band	-	chr18:3,066,786-3,220,126	7.367	73	99,2	62	41
38	*MYOZ2*	Myozenin 2	Involved in linking Z-line proteins	HCM16	chr4:120,056,920-120,108,964	2.837	23	93	198	0
39	*MYPN*	Myopalladin	Tethers together nebulin (skeletal muscle) and nebulette (cardiac muscle) to alpha-actinin, at the Z lines	DCM1KK;CMR4;HCM22	chr10:69,865,855-69,971,793	7.648	61	87,7	937	671
40	*NEBL*	Nebulette	Binds actin and interacts with thin filaments and Z-line associated proteins	-	chr10:21,068,884-21,463,136	17.194	102	97,3	456	262
41	*NEXN*	Nexilin	Actin-binding protein	DCM1CC;HCM20	chr1:78,354,181-78,409,598	3.909	38	97,9	82	42
42	*PDLIM3*	PDZ and LIM domain 3	Involved in cytoskeletal assembly	DM1	chr4:186,421,796-186,456,732	3.400	28	96,5	118	98
43	*PKP2*	Plakophilin 2	Localizes to cell desmosomes and nuclei, regulates the signaling activity of beta-catenin, and participates in linking cadherins to intermediate filaments in the cytoskeleton	ARVC9	chr12:32,943,661-33,049,800	4.999	44	88,1	593	235
44	*PLN*	Phospholamban	Inhibitor of cardiac muscle sarcoplasmic reticulum Ca(2+)-ATPase	DCM1P;HCM18	chr6:118,869,423-118,881,607	1.794	13	83,8	290	0
45	*PRKAG2*	Protein kinase, amp-activated, gamma 2 non-catalytic subunit	Member of the AMPK gamma subunit family	WPW;GLYCOGEN STORAGE DISEASE OF HEART,LETHAL CONGENITAL;HCM6	chr7:151,253,182-151,574,336	4.773	41	87,2	610	360
46	*PTPN11*	Protein tyrosine phosphatase, non-receptor type 11	Regulates cell growth, differentiation, mitotic cycle, and oncogenic transformation	JMML;METCDS;NS1;LEOPARD SYNDROME,1	chr12:112,856,517-112,947,737	6.923	54	91,8	568	83
47	*RAF1*	V-RAF-1 murine leukemia viral oncogene homolog 1	Regulates cell division, apoptosis, differentiation and migration	NS5;LEOPARD,2	chr3:12,625,081-12,705,720	3.955	39	100	0	0
48	*RBM20*	RNA binding motif protein 20	Regulates splicing	DCM1DD	chr10:112,404,136-112,599,247	7.793	63	96,7	254	10
49	*RYR2*	Ryanodine receptor 2	Component of a calcium channel	ARVC2;CPVT1	chr1:237,205,683-237,997,308	20.565	219	95,8	855	306
50	*SCN5A*	Sodium channel, voltage-gated, type v, alpha subunit	Sodium channel subunit	SUDDEN INFANT DEATH SYNDROME;VENTRICULAR FIBRILLATION DURING MYOCARDIAL INFARCTION,SUSCEPTIBILITY TO;SSS1;LQT3;PFHB1A;BRGDA1;DCM1E;ATFB10	chr3:38,589,534-38,691,184	10.353	87	96,5	365	0
51	*SGCD*	Sarcoglycan, delta	Component of the sarcoglycan complex	DCM1L;LGMD2F	chr5:155,753,748-156,194,818	10.668	78	97,9	222	0
52	*TAZ*	Tafazzin	Mitochondrial cardiolipin transacylase	BTHS	chrX:153,639,858-153,650,083	2.535	26	98,2	45	13
53	*TCAP*	Titin-cap	Muscle assembly regulating factor. Mediates the antiparallel assembly of titin at the Z-disk	DCM1N;LGMD2G	chr17:37,821,580-37,822,827	1.043	9	100	0	0
54	*TMEM43*	Transmembrane protein 43	Nuclear envelope protein	ARVC5;EDMD7	chr3:14,166,421-14,185,200	3.822	35	98,4	61	19
55	*TMPO*	Thymopoietin	Maintaining the structural organization of nuclear envelope	DCM1T	chr12:98,909,332-98,944,177	7.393	54	85,1	1.103	0
56	*TNNC1*	Troponin c type 1	Central regulatory protein of striated muscle contraction	DCM1Z;HCM13	chr3:52,485,088-52,488,077	936	10	100	0	0
57	*TNNI3*	Troponin i type 3, cardiac	Inhibitory subunit of troponin complex: blocking actin-myosin	RCM1;HCM7;DCM2A;DCM1FF	chr19:55,663,117-	1.121	14	100	0	0

			interactions and thereby mediating striated muscle relaxation		55,669,120					
58	*TNNT2*	Troponin t type 2, cardiac	Tropomyosin-binding subunit of the troponin complex	HCM2;RCM3;DCM1D;LVNC6	chr1:201,328,123-201,346,825	2.445	24	100	0	0
59	*TPM1*	Tropomyosin 1 (alpha)	Actin-binding protein	DCM1Y;HCM3;LVNC9	chr15:63,334,819-63,364,133	4.068	36	98,9	45	0
60	*TTR*	Transthyretin	Carrier protein	AMYLOIDOSIS,HEREDITARY,TRANSTHYRETIN-RELATED;CTS1;DYSTRANSTHYRETINEMIC EUTHYROIDAL HYPERTHYROXINEMIA	chr18:29,171,711-29,179,006	1.088	10	100	0	0
61	*TXNRD2*	Thioredoxin reductase 2	Thioredoxin reductase	-	chr22:19,863,021-19,929,379	2.684	29	99,5	14	0
62	*VCL*	Vinculin	F-actin binding	HCM15;DCM1W	chr10:75,757,853-75,879,934	6.366	60	96,1	246	33
63	*TTN*	Titin/connectin	plays a key role in muscle assembly, force transmission at the Z line, and maintenance of resting tension in the I band region	CMD1G, CMH9, LGMD2J, TIBIAL MUSCULAR DYSTROPHY, TARDIVE, MYOPATHY, EARLY-ONSET, WITH FATAL CARDIOMYOPATHY	Chr 2: 179.39 – 76.7	124134	877	98,4		

Table 4. Genes included in the 62 NGS panel. The acronym, function within the cell, associated disorder(s), chromosomal location and sequencing details are given for each gene.

AMP: activated protein kinase; **ASD:** Atrial Septal Defect; **ATFB:** Atrial Fibrillation; **BMD:** Becker Muscular Dystrophy; **BRGDA1:** Brugada Syndrome 1; **BTHS:** Barth Syndrome; **CMT2B1:** Charcot-Marie-Tooth disease, axonal, type 2b1; **CPVT1:** Ventricular Tachycardia, Catecholaminergic Polymorphic, 1; **CPVT2:** Ventricular Tachycardia, Catecholaminergic Polymorphic, 2; **CTRCT16:** Cataract 16; **CTS1:** Carpal Tunnel syndrome; **DAD:** Deafness, autosomal dominant; **DCWHK:** Cardiomyopathy, dilated, with woolly hair and keratoderma; **DM1:** Myotonic Dystrophy 1; **DMD:** Duchenne Muscular Dystrophy; **EDMD:** Emery-Dreifuss Muscular Dystrophy; **FAP:** Amyloidosis, hereditary, transthyretin-related; **FPLD2:** Lipodystrophy, familial partial, 2; **FRDA:** Friedreich Ataxia 1; **HGPS:** Hutchinson-Gilford Progeria; **JMML:** Juvenile Myelomonocytic Leukemia; **LGMD:** Muscular Dystrophy, Limb-Girdle; **LQT:** Long QT syndrome; **LVNC:** Left Ventricular Noncompaction; **MADA:** Mandibulo acral Dysplasia; **METCDS:** Metachondromatosis; **MFM:** Myopathy, myofibrillar; **MPD1:** Myopathy, distal; **MPDT:** Myopathy, Distal, Tateyama type; **NS1:** Noonan syndrome 1; **PFHB1A:** Progressive familial heart block, type Ia; **PPKS2:** Keratosis Palmoplantaris Striata II; **RMD:** Rippling muscle disease; **SCPNK:** Scapuloperoneal Syndrome, Neurogenic, Kaeser type; **SFWHS:** Skin fragility-woolly hair syndrome; **SPMM:** Scapuloperoneal syndrome, myopathic type; **SSS:** Sick sinus syndrome, autosomal recessive; **VF1:** Ventricular fibrillation during myocardial infarction, susceptibility to; **WPW:** Wolff-Parkinson-White syndrome

3.2.1Next generation sequencing on Ion Torrent PGM™ System

Ion Torrent PGM™ System is based on sequencing by-synthesis where pH changes caused by the release of protons when nucleotides are incorporated into the template strand. The ion's charge is detectable by the ion sensor in the chip and as nucleotides are added, the voltage change is determined and data is displayed as a peak of voltage. These chemical signals are converted into digital information for ulterior analysis (Fig. 23).

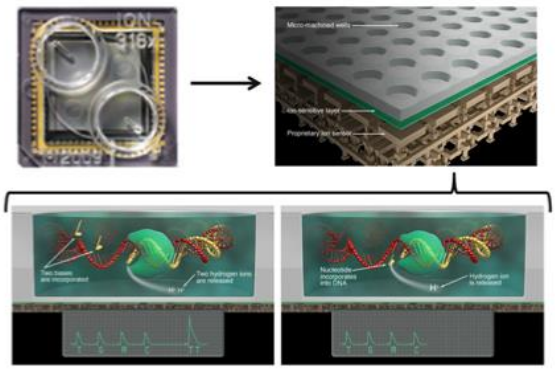


Figure 23. Ion semiconductor sequencing. As nucleotides are flowed across the chip, incorporation results in the release of a hydrogen ion that is detected by the chip. If identical nucleotides are incorporated (homopolymer), each H+ released will increase the signal detection (Images used thanks to Thermo Fisher)

This platform permits higher speed, lower cost, and smaller instrument size. It was shown that Ion Torrent PGM™ has a stable quality along sequencing reads and a good performance on mismatch accuracies, rather then in detection of indels (Liu *et al.;* 2012) (Next Generation Sequencing. Translation to Clinical Diagnostics.Lee-Jun C. Wong editor, 2013).

The fundamental strategy employed by this NGS platforms is to sequence millions of short-DNA fragments in massively parallel arrays and then realign and map the short reads back to the reference genome. The basic steps used for this work include these following steps (Fig. 24):

1. Fragmentation of selected genomic DNA into fragments of a few hundred bp;
2. Library generation by ligating adapter sequences on both ends of genomic fragments;
3. Enrichment of targeted regions of interest by multiplexed PCR-based methods;
4. Massively parallel sequencing to generate strings of bases called reads that are then aligned and assembled against the reference genome, and interpreted.

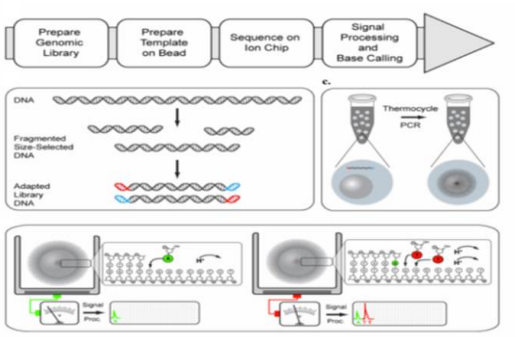


Figure 24. Ion Torrent PGM sequencing workflow (Images used thanks to Thermo Fisher)

During the first step in the workflow we generated libraries of DNA fragments flanked by the Ion Torrent adapters: during this phase, different short indexing tags called barcodes are ligated to the amplified fragment of DNA from each sample to get proper differentiation between samples (Fig. 25). The library construction process is critical to perform the most genomic coverage and obtain a good quality sequencing data (Next Generation Sequencing. Translation to Clinical Diagnostics.Lee-Jun C. Wong editor, 2013).

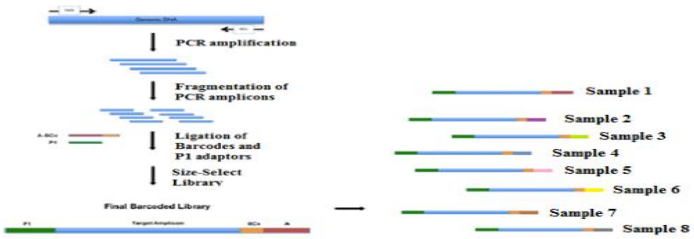


Figure 25. Library construction process for the Ion TorrentPGM™. After PCR amplification, adapters and specific barcodes are ligated to the target DNA fragments with each sample.

Once the library has been constructed, clonal amplification of each amplicon by a process called emulsion PCR (emPCR) is performed on the Ion OneTouch™ 2 System (Fig. 26), by wich single-stranded DNA fragments are attached to the surface of beads callen Ion Sphere Particles (ISPs). The basic simple of emPCR is the dilution and compartmentalization of template molecules in water droplets in a water-in-oil emulsion. Ideally, each droplet contains a single template molecule and fuction as a micro-PCR reactor (Kanagal-Shamanna *et al.;* 2016)

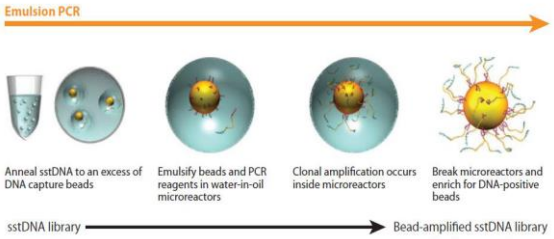


Figure 26. Schematic representation of emPCR (Images used thanks to Thermo Fisher)

After the amplification step, multiple clonal copies of the single DNA template will be obtained. Beads containing DNA are selected from those without DNA during enrichment step, which helps to maximize the sequencing yield. The enrichment template positive ISPs are loaded into a Ion chip by a short centrifugation step, and after that, the chip is placed on the PGM platform and the DNA is ready for the sequencing run (Kanagal-Shamanna *et al.;*2016).

Ion Torrent has released Ion 314, Ion 316, and Ion 318 chips (Tab. 5). The chips are different in the number of wells resulting in higher production within the same sequencing time. Ion 316 v2 chip that we used for these sequencing runsenables the production of greater than 100-400Mb data in 2 h with a read length of 200 bp. The run time also varies from 3 to 7 hours depending on the chip selected (Next Generation Sequencing Technologies in Medical Genetics, Valencia C.A., Pervaiz M.A., Husami A., Qian Y., Zhang K.C., 2013).

	Ion Torrent PGM		
Chip Type	PGM 314	PGM 316	PGM 318
# of sensors	1.3M	6.3M	11M
Total output	10-40Mb	100-400Mb	~1Gb
Run time	1-2 hrs	1-2 hrs	1-2 hrs
Read length	up to 400bp	~200bp	up to 400bp
Total reads	up to 0.6M	up to 3M	up to 6M

Table 5. Commercial chips for Ion Torrent PGM (Images used thanks to Thermo Fisher)

During the sequencing run, dNTPs are released in order and diffuse to each well. Once a nucleotide is incorporated into the growing strand, a hydrogen ion is released, leading to a pH change due to its positive charge. Changes in pH are converted into voltage alterations and the electrical signals will be processed and translated into sequence reads. The number of released ions is proportional to that of the incorporation events. When two identical nucleotides are incorporated, two hydrogen ions will be released and the reading of the digital signal will also double. But if the released nucleotide is not complementary to the template,

no hydrogen ion will be generated and the pH in solution will not change, indicating there is no incorporation event. Once data is generated on the Ion Torrent PGM™, it is automatically transferred to the Torrent Serve and it run through signal processing and base calling alghoritms that produce a lot of DNA sequences associated with individual reads.

3.3 ANALYSIS OF NGS DATA: VARIANT CALLING, ANNOTATION AND PRIORITIZATION

The data analysis of the total number and type of DNA variants identified for each patient was carried out bythe software Torrent Suite 4.0.Subsequently, the bioinformatics workflow adopted to assess the biomedical impact of the DNA variants resulting from the experimental study was defined by these points:

I. Alignment to reference genome (reference assembly GRCh37/hg19)
II. Coverage analysis (*Coverage analysis plugin*)
III. Variant Calling (*Variant Caller plugin*)
IV. Annotation of identified variants

Variant calls were annotated by wANNOVAR Web Server (wannovar2.usc.edu/). The nucleotide variants with a Minor Allele Frequency (MAF) ≤0.01 both in the 1000 Genome Project (global and European) and in the NHLBI-ESP 6500 exome project (global) were filtered and designed as "rare variants".

3.4 SANGER VALIDATION AND *IN SILICO* ANALYSIS OF NEXT GENERATION SEQUENCING VARIANTS

Sanger sequencing was the goal standard for validating DNA sequencing results from NGS analysis: between the selected variants, only the exonic and ±10 bp intronic ones were prioritized and validated by Sanger sequencing. The confirmed DNA changes were subjected to different in silico predictions, twelve methods were employed for assessing the possible pathogenicity of the identified missense changes (Tab. 9). The effect of missense changes on the structure and function of a human protein was predicted by: (i) SIFT (Sorting Intolerant From Tolerant), (ii) PolyPhen-2 (Polymorphism Phenotyping v2) HDIV, that identifies human damaging mutations by assuming differences between human proteins and their closely related mammalian homologs as non-damaging; (iii) PolyPhen-2 HVAR, that identifies human disease-causing mutations by assuming common human nsSNPs as non-damaging; (iv) Provean (Protein Variation Effect Analyzer); (v) LRT (Likelihood Ratio Test) that identifies conserved amino acid positions and deleterious mutations using a comparative genomics data set of multiple vertebrate species; (vi) Mutation Taster; (vii) Mutation Assessor; (viii) FATHMM (Functional Analysis through Hidden Markov Models); (ix) RadialSVM (Radial Support Vector Machine); (x) LRT (Logistic Regression Test);

(xi) CADD v1.3 (Combined Annotation–Dependent Depletion), a method for objectively integrating many diverse annotations into a single measure (C score) for each variant; and (xii) molecular modeling (Bottillo *et al.;* 2016a) (Bottillo *et al.;* 2016b).

3.5 MOLECULAR MODELING

Protein modelswere built using the homology modeling approach implemented in modeler-9 package on these specific missense variants: *MYBPC3* c.T1664C:p.M555T, *MYBPC3* c.C1112A:p.P371Q, *RYR2* c.A3380G:p.E1127G, *RYR2* c.G145A:p.R485Q, *FHL2* c.G109T:p.A37S, *MYOM1* c.G2132A:p.R711H, *MYOM1* c.C2131T:p.R711C, *DSG2* c.A208G:p.I70V, *CALR3* c.A1036G:p.I346V, *ABCC9* c.G2200A:p.V734I, e *ILK* c.G1086C:p.Q362H. PSI-BLAST was used to find suitable structural templates for each sequence to model and the sequences of each protein target to model and its structural template were then aligned by using the program CLUSTALW. The model displaying the lowest objective function (Burke *et al.;* 1999), which measures the extent of violation of constraints from the structural templates, was taken as the representative model. Superimposition and root-mean-square deviation (RMSD) calculation of Cα traces of the 10 models were performed to detect the most variable and therefore less reliable modeled regions. These invariably corresponded to loop elements. Procheck (Laskowski *et al.;* 1996) was used to monitor the stereochemical quality of the representative models, whereas ProsaII (Sippl *et al.;* 2014) was used to measure the overall protein quality in packing and solvent exposure.

3.6 STATISTICAL ASSESSMENT OF GENOTYPE-PHENOTYPE CORRELATIONS

All statistical analyses were performed using SPSS (statistical software package version 20.0). For each patient, the clinical data and molecular data (i.e. total number nsSNVs, number of any class of nsSNVs) were tabulated (Tab.10).

Phenotype data were presented as continuous variables obtained from clinical data and instrumental measurements. Categorical variables were shown as the presence/absence or grade, of the clinical feature. Continuous variables were summarized using means and standard deviations, while categorical variables were summarized using frequencies and percentages.

The molecular data were presented as number of DNA rare (frequency ≤ 0.01) nsSNVs variants (non-synonimus single nucleotide variants) identified in each functional class. The genes carrying rare variants were indeed grouped into sarcomeric, desmosomal, K+ and Na+ channels and

interacting proteins, loci for mRNA splicing and cellular enzymes, cytoskeletal, Z-disk and Ca^{2+} homeostasis.

Statistical correlations for non-syndromic patients were evaluated between the presence of rare DNA variants (or the family history) and the prevalence of phenotypic traits with the following methods: (i) Chi-square test when referred to categorical variables; (ii) unpaired two-tailed Student's t-test when related to continuous variable. Due to the relatively low number of patients no adjustments was planned for multiple testing; the analysis is therefore exploratory and results to be considered as hypotheses-generating.

3.7 ANALYSIS OF THE X CHROMOSOMES METHYLATION STATUS

Case 27 was affected by Danon's disease and an assay for evaluating the methylation status (i.e., inactivation) of the X chromosomes (XCI) was also investigated on DNA both from blood leukocytes and cardiac muscle obtained from the LV, RV, and the septum of the patient's explanted heart. The same test was also performed on her father's DNA in order to establish the parental inheritance of each X chromosome.The XCI pattern was determined by evaluating the cytosine methylation of CpG dinucleotides within the polymorphic Cytosine, Adenine and Guanine (CAG) repeat in the first exon of the androgen receptor (AR) gene, located on chromosome X (Elstein et al.; 2012) DNA was digested by methylation-sensitive restriction enzymes (i.e., HpaII). Digestion protocol was characterized by the preparation of two samples of 500 ng DNA: one of them containing 10U of the methylation-sensitive restriction enzyme HpaII and its specific digestion tampon, and the other one containing only the digestion tampon (Tab.6).

	Digestion Mix A	**Digestion Mix B**
DNA (500ng)	500/[quantification]	500/[quantification]
Buffer tango (10x)	2 µl	2 µl
HpaII	1µl	____
H2O	to volume	to volume
Vf	20 µl	20 µl

Table 6. Digestion Mix for the analysis of the X chromosomes methylation status

Both digestion mixes were incubated overnight at 37° C, after that the enzyme was inactivated at 65° for 20 minutes. After digestion, DNA amplification can occur only in presence of methylated restriction sites (inactive allele). Both a digested and an undigested DNA samples were then amplified by two different primer pairs specific for the AR locus.
The samples were than amplified with PCR, using two couples of oligonucleotide specific for the AR locus:

Couple 1:
XinF1 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3' marked with 5′ FAM
XinR1 5'-TCCAGAATCTGTTCCAGAGCGTGC-3'

Couple 2:
XinF2 5'-GTGCGCGAAGTGATCCAGAA-3' marked with 5′ HEX
XinR2 5'-CCAGGACCAGGTAGCCTGTG-3'

Each sample was amplified in the Applied Biosystems 9700 Fast Thermal Cycler (Applied Biosystems, Foster City, CA) and run in duplicate.

PCR Reaction Mix (Vf = 25µl)

- PCR BUFFER 10X: 2.5µl
- MgCl2 (25Mm): 1.5µl (conc. 1.5mM)
- dNTPs (2mM): 2.5µl (conc. 200µM)
- PRIMER F (10µM): 1µl
- PRIMER R (10µM): 1µl
- DMSO: 2,5 µl (10X)
- FastStart Taq DNA 5U/µl: 0,125µl
- Sterile distilled H2O: to volume
- DNA (from digestion): 4µl

PCR conditions:

Initial denaturation: 95°c 4 min
Cyclical denaturation: 95° 40 sec
Annealing: 59°c 40 sec
Cyclical extension: 72°c 40 sec
Final extension: 72°c 30 min

Cycles: 30

Polymerase Chain Reaction (PCR) products were separated an automated sequencer ABI3500xL (Thermo Fisher Scientific). GeneMapper 5 software (Thermo Fisher Scientific) was used for quantification and interpretation of raw data output. Peak heights of fluorescence intensity were used to calculate ratios. All samples were analyzed in triplicate, and the average values were used to assess the degree of X inactivation. The XCI skewing ratio was determined by comparing the ratio of allele peak heights in the digested sample (d1 and d2, for paternal and maternal allele, respectively) with the ratio in the undigested sample (u1 and u2), according to the following proportion:

$$\text{XCI percentage of the paternal allele}=[(d1/u1)/\{(d1/u1)+(d2/u2)\}]\times 100,$$

which ranges from 0% to 100% (Wardburton *et al.;* 2009).If this ratio is greater than or equal to 80/20 the X inactivation is highly biased to allele 1; if greater than or equal to 75/25 the X inactivation is biased to allele 1; if about 50/50 the X inactivation is random. If a random X inactivation occurs, both alleles would be amplified; otherwise, if a preferential X inactivation occurs for the active chromosome's allele, not methylated, it will be completely digested from the methylation-sensitive enzyme and only the other allele would be amplified.

4. RESULTS

4.1 GENOTYPING RESULTS

Forty-four unrelated Caucasian/Italian patients (23 males and 21 females) were included in this study. Three patients displayed hypertrophic cardiomyopathy associated with a syndromic phenotype: case 10, 24 and 27 were respectively affected by a mild form of Fabry's disease, by Cantù's syndrome and by Danon's disease.

High numbers, such as 3x106 reads (~700.000 reads) was obtained from the 62 genes NGS panel and 100x106 bp were aligned on the reference genome (reference assembly GRCh37/hg19). Each reads has a mean read length of 140 bp. Among the 44 patients the mean read depth of each analysed gene was 222X (Fig.27).

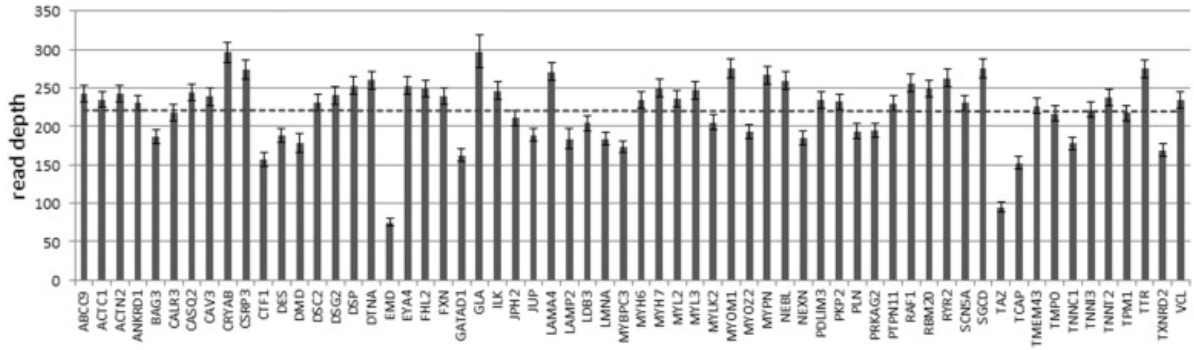


Figure 27. Schematic representation of the read depth of 62 genes included in NGS panel (Bottillo *et al.;* 2016a, Supplementary Table 1)

The total number of identified variants, before filtering for each patients is shown in Table 7.

Subsequently the total number of the DNA variants was filtered and underwent Sanger sequencing. Among the 41 non-syndromic HCM cases, 33 (80%) were found to carry at least one rare non-synonimus single nucleotide variants (nsSN). In total, 95 non-synonymous sequence changes, of which 87 different, were identified. These included 73 missense, 13 intronic, 3 frameshift, 4 nonsense, one stop-loss and one in frame deletion. Among the 87 different nsSNVs, 21 were already reported in literature (Tab. 8).

Pt	Exonic	Splicing (±2bp)	Intronic	UTR	ncRNA	TOTAL
1	78	3	218	142	2	443
2	84	2	212	139	1	438
3	76	0	182	122	3	383
4	94	2	208	135	4	443
5	81	1	186	138	4	410
6	90	0	220	100	2	412
7	79	0	205	110	0	394
8	78	0	204	148	2	432
9	82	1	183	151	0	417
10	91	0	209	166	2	468
11	80	0	184	102	0	366
12	91	1	205	156	3	456
13	90	0	225	145	4	464
14	72	0	178	134	2	386
15	95	0	184	119	2	400
16	87	0	183	110	0	380
17	90	0	176	116	2	384
18	74	0	199	116	0	389
19	91	0	197	165	0	453
20	91	0	216	116	2	425
21	93	1	195	162	1	452
22	92	0	207	130	2	431
23	76	1	205	106	2	390
24	99	1	209	141	2	452
25	85	1	214	152	9	461
26	90	2	206	142	7	447
27	99	1	195	157	2	454
29	99	0	207	127	3	436
31	96	0	200	146	2	444
32	89	1	201	149	0	440
33	88	0	212	127	2	429
34	98	0	215	129	0	442
35	92	0	205	144	4	455
36	86	0	213	146	14	459
37	87	0	194	131	11	423
38	76	1	204	153	9	443
43	90	0	193	93	10	386
44	104	0	198	107	12	421
45	93	0	208	122	2	425
46	90	1	204	146	9	450
49	95	0	225	151	9	480
51	93	0	202	108	11	414
52	88	1	197	98	6	390
55	92	0	210	131	10	443
TOTAL	**3884**	**21**	**8893**	**5828**	**174**	**18810**

Pt: patient; **UTR**: untraslated region

Table 7. Numbers of DNA point mutations in 44 patients with hypertrophic cardiomyopathy (Bottillo *et al.;* 2016a, Supplementary Table 2)

The proportion of non-synonimus single nucleotide variants (nsSNVs) identified for each analyzed gene is shown in Figure 28a.

Some patients showed the same eight nsSNVs:

- *CAV3*c.C233T, p.T78M (patients 17,38);
- *CSRP3*c.T10C, p.W4R (patients 8,25);
- *DSC2*c.C1787T, p.A596V (patients 11,23);
- *MYBPC3* c.3192dupC, p.K1065fs (patients 31,46);
- *MYBPC3* c.T1664C, p.M555T (patients 1,51);
- *PKP2*c.G76A, p.D26N (patients 14,34);
- *PKP2* c.C2299A, p.R767S (patients 24,31);
- *RBM20*c.G3373A, p.E1125K (patients 8, 25, 49).

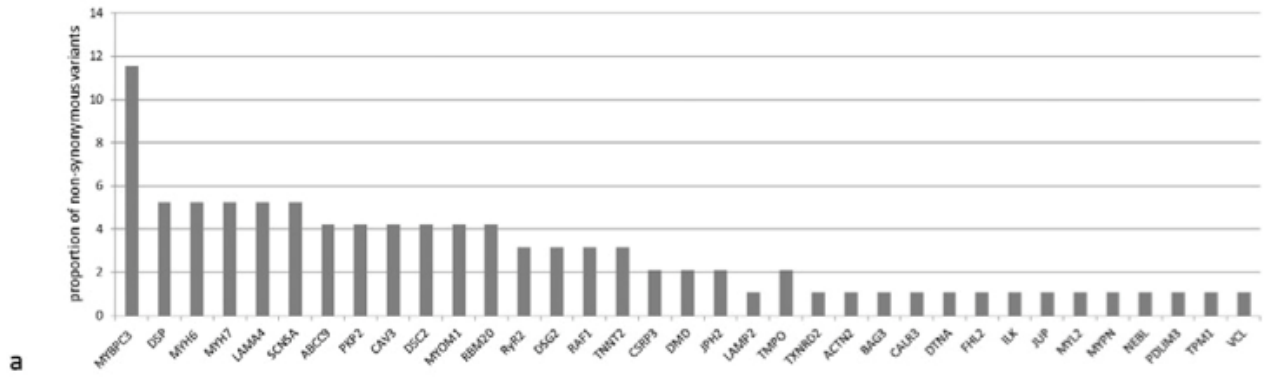


Figure 28a. Proportion of nsSNVs identified for each analyzed gene (Bottillo *et al.;* 2016a, Supplementary Table 1)

Pt	Gene	Mutation	Status	1000G ALL	1000G EUR	ESP6500si ALL	Type	ClinVar SIG	Reference	Comment
1	*MYBPC3*	c.T1664C,p.M555T	Htz	–	–	–	missense	unk	Girolami et al. 2006	No predicted deleterious effect
	RyR2	c.A3380G,p.E1127G	Htz	0.0006	–	0.0006	missense	unk	–	8/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
3	*TMPO*	c.A1037G,p.H346R	Htz	0.0002	–	–	missense	–	–	No predicted deleterious effect
5	*MYH6*	c.643–5C>T	Htz	–	–	0.0005	intronic	unklPnPath	–	Branch–point splice site broken
8	*RBM20*	c.G3373A,p.E1125K	Htz	0.002	0.007	0.0037	missense	PnPath	–	Ambiguous *in silico* predictions
	MYH6	c.2928+5G>A	Htz	0.0002	0.001	0.0018	intronic	PnPath	–	Donor splice site broken
	CSRP3	c.T10C,p.W4R	Htz	0.0018	0.005	0.0037	missense	unklPnPath	Knoll et al. 2002 Geier et al. 2008	6/12 deleterious predictions Previously reported alone in CMD1M patients and in conjunction with a sarcomeric mutation in HCM patients The reference allele is conserved across evolutionarily distant species
	LAMA4	c.A4937G,p.E1646G	Htz	–	–	0.0001	missense	–	–	11/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
	LAMA4	c.G1565C,p.R522T	Htz	–	–	–	missense	–	–	No predicted deleterious effect
9	*MYBPC3*	c.A3825C,p.*1275Cysext*33	Htz	–	–	–	stop loss	unk	–	Stop loss
	NEBL	c.G604A,p.G202R	Htz	0.0014	0.003	0.0018	missense	–	Purevjav et al. 2010	Previously identified in one DCM individual. The Gly202Arg mouse model exhibits DCM features
	RyR2	c.9450–9C>T	Htz	–	–	–	intronic	–	–	No predicted deleterious effect
10*	*MYBPC3*	c.C1112A,p.P371Q	Htz	–	–	–	missense	Path	–	8/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
	GLA	c.A644G,p.N215S	Htz	–	–	–	missense	–	Eng et al. 1993 Davies et al. 1993	9/12 deleterious pr edictions Previously associated with a mild forms of Fabry disease The reference allele is conserved across evolutionarily distant species
	DSP	c.G137A,p.G46D	Htz	–	–	0.0001	missense	–	–	No predicted deleterious effect
	TXNRD2	c.G1150A,p.G384S	Htz	0.0018	0.006	0.0041	missense	–	Sibbing et al. 2011	Ambiguous *in silico* predictions
11	*JPH2*	c.G1536C,p.W512C	Htz	–	–	–	missense	–	–	Ambiguous *in silico* predictions
	SCN5A	c.A5605T,p.I1869F	Htz	–	–	–	missense	PnPath	–	12/12 deleterious predictions
	FHL2	c.G109Tp.A37S	Htz	0.0016	–	0.0023	missense	unk	–	Ambiguous *in silico* predictions
	DSC2	c.C1787T,p.A596V	Htz	0.0022	0.002	0.0009	missense	PnPath	den Haan et al. 2009	Ambiguous *in silico* predictions Previously reported in one individual with ARVC The reference allele is conserved across evolutionarily distant species
14	*PKP2*	c.G76A,p.D26N	Htz	0.003	0.011	0.0049	missense	–	Christensen et al. 2010	6/12 deleterious predictions Previously reported as modifier of disease, over–represented in ARVC cases
15	*MYOM1*	c.C2131T,p.R711C	Htz	–	–	0.0001	missense	–	–	11/12 deleterious predictions
	RAF1	c.[124G>A;125C>T] p.A42I	Htz	–	–	–	missense	–	–	Ambiguous *in silico* predictions

19	*DSP*	c.4441_4443del,p.K1481_1481del	Htz				in frame del	–	–	In frame deletion
	MYOM1	c.G2132A,p.R711H	Htz	0.0002	–	–	missense	Ppath	–	11/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
	SCN5A	c.C5837T,p.S1946F	Htz	–	–	–	missense	–	Hermida JS 2010	7/12 deleterious predictions Reported as "probably pathogenetic" in ClinVar database
	DSC2	c.C2328G,p.I776M	Htz	–	–	0.0001	missense	–	–	No predicted deleterious effect
	LAMA4	c.T3482C,p.M1161T	Htz	–	–	–	missense	–	–	No predicted deleterious effect
	LAMP2	c.C418G,p.L140V	Hem	–	–	–	missense	–	–	No predicted deleterious effect
20	*DSG2*	c.G2147A,p.G716E	Htz	–	–	–	missense	nPath	–	No predicted deleterious effect
	LAMA4	c.4665+8G>T	Htz	0.0004	–	0.0016	intronic	otherlPath	–	IIE site splice broken
21	*MYBPC3*	c.2309–2A>G	Htz	–	–	–	intronic	–	Van Driest 2004 Roncarati 2011	Acceptor splice site broken Reported as "pathogenetic" in ClinVar database
	RyR2	c.G1454A,p.R485Q	Htz	–	–	–	missense	PPathlnPath	–	10/12 deleteri ous predictions The reference allele is conserved across evolutionarily distant species
	ABCC9	c.G2200A,p.V734I	Hmz	0.004	0.014	0.0092	missense	–	Minoretti et al. 2006	Ambiguous *in silico* predictions Previously reported as influencing susceptibility to precocious myocardial infarction
	CALR3	c.A1036G,p.I346V	Htz	–	–	–	missense	PnPathlother	–	No predicted deleterious effect
	TNNT2	c.264+7G>A	Htz	0.01	–	0.011	intronic	–	Kassem et al 2013	Donor splice site broken. It is a common variant in the Black population
22	*MYH7*	c.4402G>A,p.E1468K	Htz	–	–	–	missense	–	–	9/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
	ILK	c.G1086C,p.Q362H	Htz	–	–	0.0001	missense	unk	–	11/12 deleterious predictions
23	*DSC2*	c.C1787Tp.A596V	Htz	0.0022	0.002	0.0009	missense	–	den Haan et al. 2009	Ambiguous *in silico* predictions Previously reported in one individual with ARVC The reference allele is conserved across evolutionarily distant species
	LAMA4	c.G1967A,p.S656N	Htz	–	–	–	missense	–	–	No predicted deleterious effect
	MYH6	c.G68A,p.R23H	Htz	–	–	–	missense	–	–	11/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
	TPM1	c.C134T,p.A45V	Htz	–	–	–	missense	unklunk	–	9/12 deleterious predictions The reference allele is conserved across evolutionarily distant species

24*	*PKP2*	c.C2299A,p.R767S	Htz	0.0006	0.002	0.0004	missense	Pathlother	Fressart 2010 Klauke 2010 Tan 2010	6/12 deleterious predictions Previously identified in 4 ARVC, one HCM, and one VT patients
	DSP	c.G88A,p.V30M	Htz	0.0022	0.001	0.0012	missense	–	Yang et al. 2006	Previously found in one ARVC patient. The mutant allele fails to localize to the cell membrane in a desmosome–forming cell line and fails to bind to and coimmunoprecipitate junction plakoglobin
	RyR2	c.A4273G,p.T1425A	Htz	–	–	–	missense	–	–	Ambiguous *in silico* predictions The reference allele is conserved across evolutionarily distant species
	MYBPC3	c.821+3G>A	Htz				intronic	Path	–	IIE splice site broken
	ABCC9	c.C3460T,p.R1154W	Htz	–	–	–	missense	–	van Bon 2012 Harakalova 2012	11/12 deleterious predictions Reported as "pathogenetic" in ClinVar database Causes Cantù syndrome
25	*MYBPC3*	c.2846dupT,p.M949fs	Htz	–	–	–	frameshift	unk/PnPath	–	Truncating mutation
	TXNRD2	c.375-8C>T	Htz	0.0004	–	0.0007	intronic	PnPath	–	No predicted deleterious effect
	CSRP3	c.T10C,p.W4R	Htz	0.0018	0.005	0.0037	missense	unk	Knoll et al. 2002 Geier et al. 2008	Previously reported alone in CMD1M patients and in conjunction with a sarcomeric mutation in HCM patients
	RBM20	c.G3373A,p.E1125K	Htz	0.002	0.007	0.0037	missense	unk	–	Ambiguous *in silico* predictions
26	*DSP*	c.C3956T,p.T1319I	Htz	–	–	0.0001	missense	untested	–	8/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
	ABCC9	c.2238-1G>A	Htz	0.0018	0.001	0.0008	intronic	otherlPath	–	Acceptor splice site broken The reference allele is conserved across evolutionarily distant species
	CAV3	c.C233T,p.T78M	Htz	0.002	0.005	0.0043	missense	–	Fulizio et al. 2005 Vatta et al 2006 Cronk et al 2007	8/12 deleterious predictions Previously found in 3 unrelated individuals with long QT syndrome. The mutant causes a 5–fold increase in late sodium current compared to wild–type
	MYBPC3	c.2309–2A>G	Htz	–	–	–	intronic	–	–	Acceptor splice site broken Reported as "pathogenetic" in ClinVar database
	DTNA	c.C2095T,p.R699C	Htz				missense	PnPath	–	No predicted deleterious effect
27*	*LMNA*	c.1363–7T>C	Htz				intronic	unklunk	–	No predicted deleterious eff ect
	LAMP2	c.453delT,p.F151fs	Hem				frameshift	PathlnPath	–	Truncating mutation
29	*ABCC9*	c.816+11G>A	Htz	0.0004	0.001	0.0002	intronic	unk/PnPathlnPath	–	Donor splice site broken
31	*PKP2*	c.C2299A,p.R767S	Htz	0.0006	0.002	0.0004	missense	–	Fressart 2010 Klauke 2010 Tan 2010	6/12 deleterious predictions Previously identified in 4 ARVC, one HCM, and one VT patients
	JPH2	c.G1513A,p.G505S	Htz	0.015	0.011	0.0071	missense	–	Matsushita 2007 Bean 2013	Previously identified in 4 Japanese HCM probands
	MYBPC3	c.3192dupC,p.K1065fs	Htz	–	–	–	frameshift	–	Girolami 2006 Girolami 2010 Olivotto 2011	Truncating mutation
32	*JUP*	c.909+6C>T	Htz	–	–	0.0014	intronic	–	–	No predicted deleterious effect
	RAF1	c.G1858A,p.A620T	Htz	–	–	–	missense	PnPath	–	6/12 deleterious pre dictions The reference allele is conserved across evolutionarily distant species
	SCN5A	c.C1810G,p.L604V	Htz	–	–	–	missense	unk/PPath	–	Ambiguous *in silico* predictions
33	*PDLIM3*	c.G163T,p.E55X	Htz	–	–	–	nonsense	–	–	Truncating mutation
	MYBPC3	c.C3775Tp.Q1259X	Htz	–	–	–	nonsense	PnPath	–	Truncating mutation

35	*MYPN*	c.C3335T,p.P1112L	Htz	0.0026	0.005	0.0021	missense	PPath/Path	Dubosc q–Bidot 2007 Adzhubei 2010 Purevjav 2012	7/12 deleterious predictions Previously reported in HCM and DCM patients The reference allele is conserved across evolutionarily distant species
	CAV3	c.G221A,p.R74H	Htz	0.0002	–	–	missense	PnPath	El Huneidi 2014	11/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
36	*MYH7*	c.G676A,p.A226T	Htz	–	–	–	missense	–	–	9/12 deleterious predictions
37	*DSP*	c.G6188A,p.R2063Q	Htz	–	–	0.0001	missense	unk	–	Ambiguous *in silico* predictions
	DSC2	c.C595T,p.R199C	Htz	–	–	–	missense	PnPath	–	Ambiguous *in silico* predictions
38	*CAV3*	c.C233T,p.T78M	Htz	0.002	0.005	0.0043	missense	other/Path	Falizio et al. 2005 Vatta et al 2006 Cronk et al 2007	8/12 deleterious predictions Previously found in 3 unrelated individuals with long QT syndrome. The mutant causes a 5–fold increase in late sodium current compared to wild–type
	MYH7	c.G3346A,p.E1116K	Htz	–	–	–	missense	PnPath/unk	–	10/12 deleterious predict ions Reported as "pathogenetic" in ClinVar database The reference allele is conserved across evolutionarily distant species
43	*MYH6*	c.G622A,p.D208N	Htz	0.002	0.005	0.0049	missense	other/Path	Granados-Riveron et al. 2010	No predicted deleterious effect
	MYBPC3	c.C1302A,p.Y434X	Htz	–	–	–	nonsense	PnPath	–	Truncating mutation
44	*MYH6*	c.G4193A,p.R1398Q	Htz	–	–	0.0001	missense	–	–	7/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
	DSG2	c.G2033C,p.G678A	Htz	–	–	0.0002	missense	nPath	–	No predicted deleterious effect
	PKP2	c.C1445T,p.T482M	Htz	0.0038	0.005	0.0037	missense	unk	–	No predicted deleterious effect
	MYH7	c.G428A,p.R143Q	Htz	–	–	–	missense	unk	Song 2005 Van Driest 2004 Wang 2007 Kimura 2010 Gruner 2011 Fokstuen 2011	9/12 deleterious predictions Previously reported in 6 HCM individuals The reference allele is conserved across evolutionarily distant species

45	*MYBPC3*	c.A649G,p.S217G	Htz	0.0018	0.001	0.001	missense	unk	Lakdawala et al. 2012	Ambiguous *in silico* predictions Likely benign when present in isolation
	MYH7	c.C2167T,p.R723C	Htz	–	–	–	missense	unk/PnPath	Watkins 1992 Tesson 1998 Richard 2003 Ingles 2005 Girolami 2010	10/12 deleterious predictions Previously reported in 5 HCM families Residue 723 is conserved among all known cardiac MHCs and all vertebrate striated muscle MHCs except the human perinatal and rabbit skeletal isoforms Mutation to a Cys changes Thr net charge
46	*MYBPC3*	c.3192dupC,p.K1065fs	Htz	–	–	–	frameshift	–	Girolami 2006 Girolami 2010 Olivotto 2011	Truncating mutation
49	*RBM20*	c.G3373A,p.E1125K	Htz	0.002	0.007	0.0037	missense	–	–	Ambiguous *in silico* predictions
	DSP	c.C5851T,p.R1951X	Htz	–	–	–	nonsense	ClinVar_SIG	–	Truncating mutation
	TMPO	c.G1696A,p.D566N	Htz	–	–	–	missense	unk	–	Ambiguous *in silico* predictions
	MYL2	c.G34T,p.G12C	Htz	–	–	–	missense	unk	–	7/12 deleterious predictions The reference allele is conserved across evolutionarily distant species
51	*VCL*	c.A1907G,p.H636R	Htz	0.0004	0.001	0.0009	missense	–	Zimmerman 2010	9/12 deleterious predictions Detected in 1/>250 Caucasian DCM individuals and in another DCM proband of unknown ethnicity The reference allele is conserved across evolutionarily distant species
	MYBPC3	c.T1664C,p.M555T	Htz	–	–	–	missense	unk/PnPath	Girolami et al. 2006	No predicted deleterious effect
52	*ACTN2*	c.C1484T,p.T495M	Htz	–	–	0.0001	missense	PnPath		Ambiguous *in silico* predictions The reference allele is conserved across evolutionarily distant species
	SCN5A	c.C2074A,p.Q692K	Htz	0.0002	0.001	0.0002	missense	PnPath	Van Langen 2003 Ackerman 2004	Ambiguous *in silico* predictions
	DMD	c.G1646A,p.R549Q	Hem	–	–	–	missense	unk/PnPath	–	Ambiguous *in silico* predictions The reference allele is conserved across evolutionarily distant species
	DMD	c.C3164T,p.T1055I	Hem	–	–	–	missense	–	–	No predicted deleterious effect

Table 8. Rare nsSNVs identified in 36/44 HCM patients. A comment inferred from review of literature, in silico predictions and evolution conservation analysis, is given for each DNA change in the last column. Based on those considerations and on the type of variant, comments with a gray background highlight DNA changes predicted to be possibly pathogenetic. Comments in bold stand for DNA variants that have been previously reported as phenotype modifying factors. Pt: patient; 1000G ALL: MAF in 1000 Genomes Project global; 1000G EUR: MAF in 1000 Genomes Project European; ESP6500si ALL: MAF in exome sequencing project global; Htz: heterozygosity; Hem: hemizygosity; Hmz: homozygosity; ClinVar SIG: signifiance in ClinVar database, including unknown (unk), non-pathogenic (nPath), probably-non-pathogenic (PnPath), probably-pathogenic (PPath), pathogenic (Path); HCM: hypertrophic cardiomyopathy; DCM: dilated cardiomyopathy; CMD1M: cardiomyopathy, dilated, 1M; ARVC: arrhythmogenic right ventricular cardiomyopathy; IIE: intron identity element; VT: ventricular tachycardia; MHC: myosin heavy chain.∗Syndromic patients (Bottillo *et al.;* 2016a)

The molecular analyses also identified 49 heterozygous synonymous variants (Tab. 9).

Pt	Gene	Synonymous variant
3	*SCN5A*	c.T1764C, p.H588H
4	*SGCD*	c.G210A, p.R70R
	TMEM43	c.C622T, p.L208L
7	*SCN5A*	c.T1587C, p.I529I
8	*MYH6*	c.G5736A, p.A1912A
10	*MYBPC3*	c.A2781G, p.T927T
11	*DMD*	c.G1650A, p.Q550Q
	RYR2	c.C3888T, p.N1296N
13	*VCL*	c.C1671T, p.D557D
	VCL	c.T1317C, p.S439S
	LMNA	c.G276A, p.L92L
14	*LMNA*	c.G276A, p.L92L
17	*MYPN*	c.A405C, p.A135A
19	*MYH7*	c.C4134T, p.D1378D
	MYOM1	c.A2658G, p.K886K
	GATAD1	c.T456C, p.G152G
20	*TPM1*	c.T519G, p.A173A
	MYH6	c.C480T, p.N160N
21	*TXNRD2*	c.1308C>T, p.F436F
23	*LAMA4*	c.T924C, p.H308H
24	*VCL*	c.T1317C, p.S439S
	LMNA	c.G276A, p.L92L
27	*DSP*	c.G4383A, p.E1461E
	MYOZ2	c.A237G, p.A79A
	MYH7	c.C1410T, p.F470F
31	*LAMA4*	c.T924C, p.H308H
33	*PRKAG2*	c.C507T, p.T169T
34	*DSG2*	c.G2340T, p.A780A
35	*DSC2*	c.A1350G, p.R450R
	TMPO	c.G1539A, p.L513L
	MYH7	c.C1410T, p.F470F
37	*DSC2*	c.A348G, p.Q116Q
38	*LAMA4*	c.T924C, p.H308H
43	*LDB3*	c.T546C, p.S182S
	RYR2	c.C1863T, p.H621H
44	*EYA4*	c.G714A, p.T238T
	VCL	c.T1317C, p.S439S
	ABCC9	c.C2523T, p.A841A
45	*SCN5A*	c.G744A, p.K248K
	MYH7	c.C1410T, p.F470F
46	*MYH7*	c.C1410T, p.F470F
25	*LDB3*	c.G273A, p.T91T
49	*RBM20*	c.G1161A, p.A387A
	RyR2	c.G13740A, p.T4580T
51	*DSP*	c.G105A, p.G35G
52	*TMEM43*	c.G333A, p.P111P
	ANKRD1	c.T496C, p.L166L
55	*LDB3*	c.G273A, p.T91T
	DMD	c.G1023T, p.V341V

Table 9. Numbers of heterozygous synonymous variants in 44 patients with hypertrophic cardiomyopathy (Bottillo *et al.;* 2016a, Supplementary Table 3)

No nsSNVs were identified between 25/62 genes: *ACTC1, ANKRD1, CASQ2, CRYAB, CTF1, DES, EMD, EYA4, FXN, GATAD1, LDB3, MYL3, MYLK2, MYOZ2, NEXN, PLN, PRKAG2, PTPN11, SGCD, TAZ, TCAP, TMEM43, TNNC1, TNNI3* and *TTR*.

About 33% of the changes mapped in sarcomeric loci, 19% in desmosomal loci, 15% in genes coding for K+ and Na+ channels and for channels-interacting proteins, 12% in genes coding for mRNA splicing and other cellular enzymes, 9% in cytoskeletal loci, 6% in genes related to intracellular Ca++ homeostasis, 5% in Z-disk loci and 5% in other non-sarcomeric loci (Fig. 28b). The 27% of the patients harbored two heterozygous DNA changes in different genes, 15% a single heterozygous change and 39% three or more variants (Fig. 28c).

Twenty-four/41 of the cases harbored at least one sarcomeric nsSNV, and three patients carried only desmosomal changes. In total, 58% carried alterations in sarcomeric loci, 14% in desmosomal and 7% in other non-sarcomeric ones without any sarcomere change.

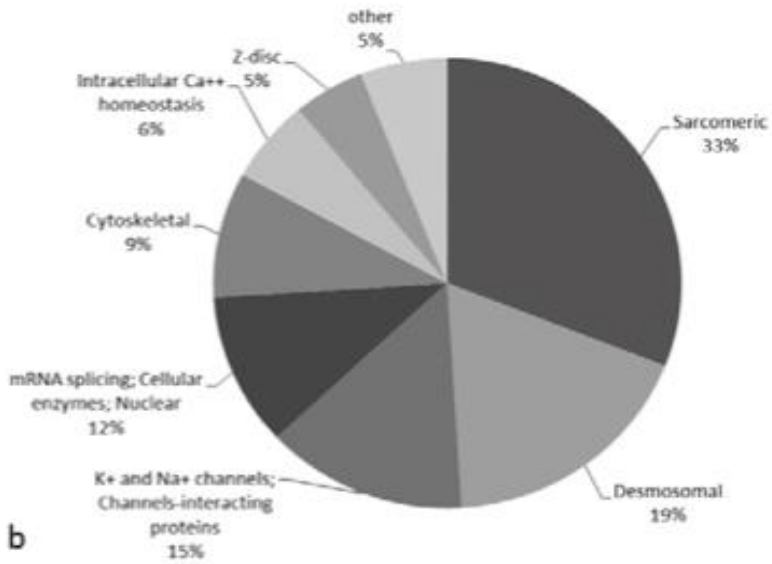


Figure 28b. Proportion of nsSNVs identified for for each functional genic category. "Other" includes the loci coding for lysosomal, thioredoxin reductase, anti-apoptotic and membrane proteins (Bottillo *et al.;* 2016a)

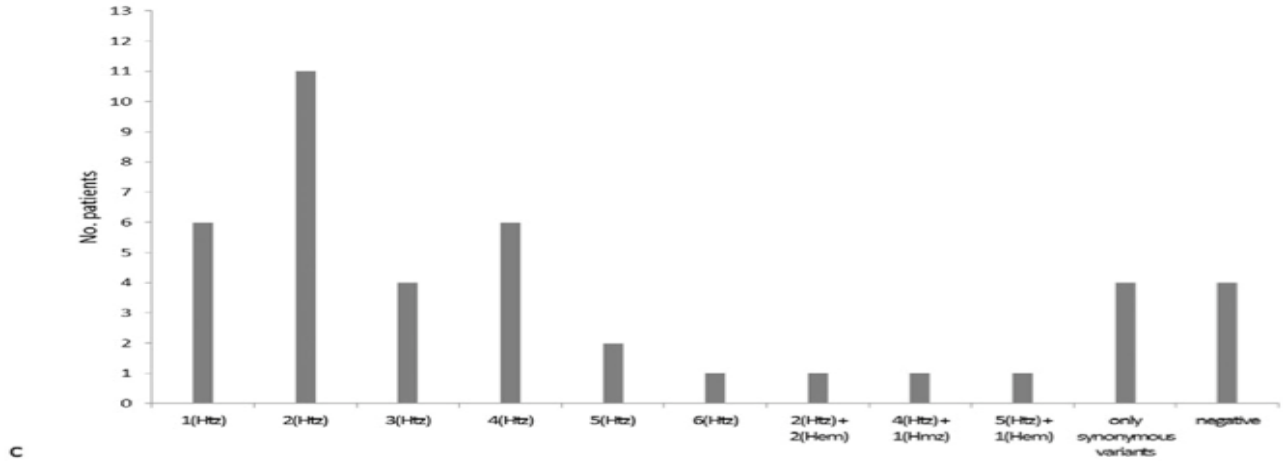


Figure 28c. Number and zygosity of DNA changes identified over 37 HCM patients. Six cases were found to carry a single heterozygous nsSNV, 11 cases two heterozygous nsSNVs and 16 cases three or more nsSNVs. Four patients carried only synonymous changes, and four resulted negative to the NGS screening (Bottillo *et al.;* 2016a)

Twenty-four/41 of the cases harbored at least one sarcomeric nsSNV, and three patients carried only desmosomal changes. In total, 58% carried alterations in sarcomeric loci, 14% in desmosomal and 7% in other non-sarcomeric ones without any sarcomere change. Figure 28d illustrates the fraction of mutated cases respect to the different genes' categories.

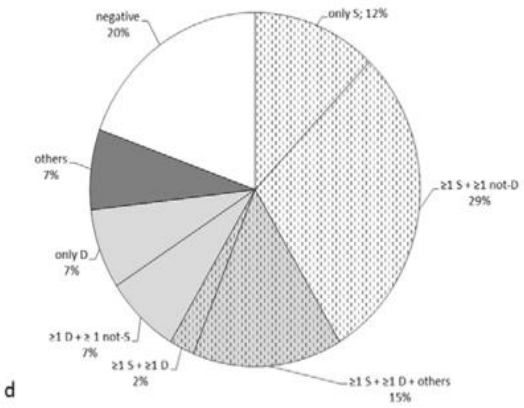


Figure 28d. Proportion of mutated patients respect to the genes' category. Dashed slices (both white and gray) stand for cases harboring at least one sarcomeric non-synonymous variant. Light gray slices (both dashed and not dashed) represent patients not carrying any sarcomeric nsSNV, but at least one desmosomal one (Bottillo *et al.;* 2016a)

In particular only one sarcomeric nsSNV was identified in patients 5, 36 and 46 and two sarcomric nsSNVs in patients 43 and 45.

Seven patients (19, 23, 26, 31, 49, 10, 24) carried out at least, one sarcomeric nsSNVs associated

with one desmosomal variants and other variants.

Case 44 showed two sarcomeric changes and two desmosomal nsSNVs.

Instead 18 patients didn't show any sarcomeric nsSNVs and among them, patients 14 and 37 showed only desmosomal changes; patients 3, 27, 29 and 52 carried out only variants in other genes.

The same 44 unrelated Caucasian/Italian patients affected by hypertrophic cardiomyopathy were analyzed by another NGS panel: the second panel was designed to analyze coding, intronic junctions and UTR sequences of *TTN* genes. Two thousand and five variants were identified in these patientsanalyzed by the*TTN* panel: 1588 exonic changes, 480 intronic changes, 20 UTR variants and 517 genetic variants in non-coding RNAs (ncRNAs) (Fig. 29).

Figure 30shows the total number of DNA variantsdetected by *TTN* panel for each patients.

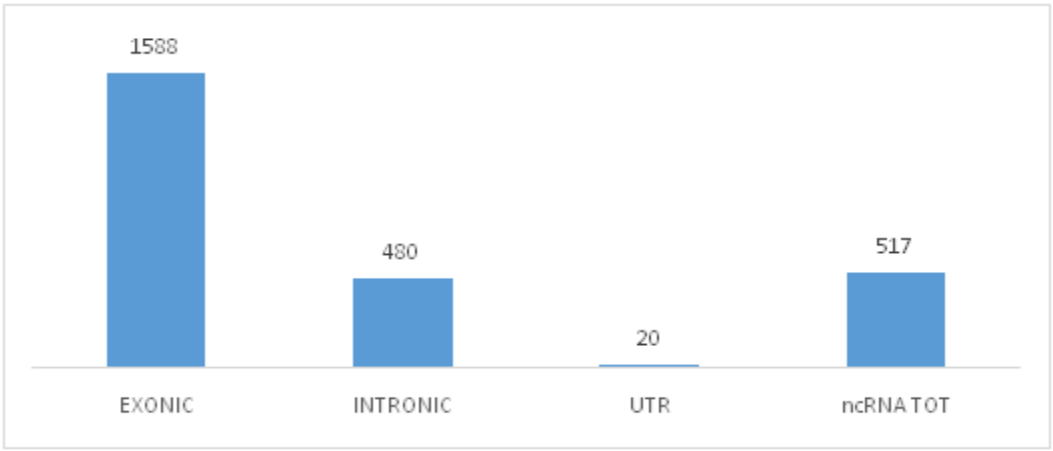


Figure 29. Variants identified in 44 HCM patients analyzed by *TTN*NGS panel

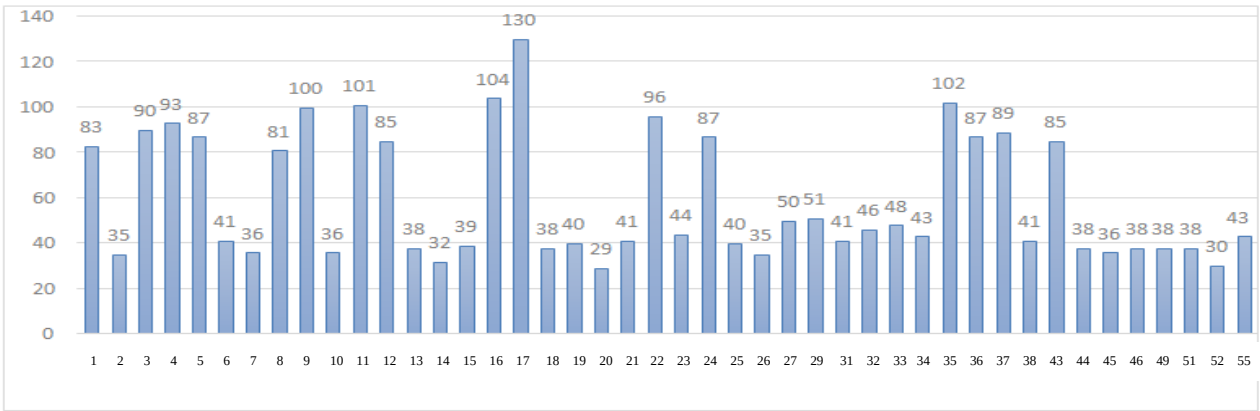


Figura 30. Total number of *TTN* variants for each 44 HCM patients

Subsequently, the total number of the titin changes was filtered with a MAF ≤ 0,05 and underwent to Sanger sequencing (Fig.31).

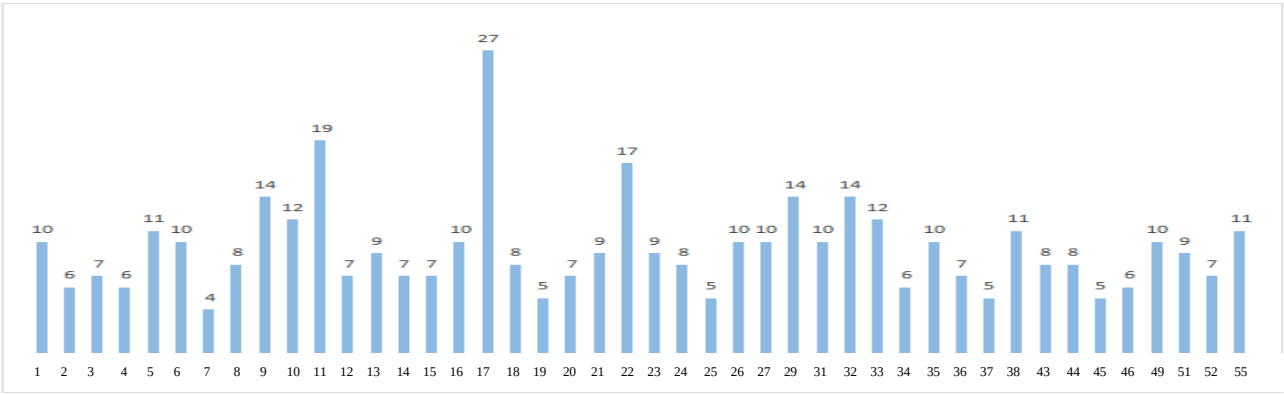


Figure 31. Total number of *TTN* variants for each 44 HCM patients after filtration (MAF ≤ 0,05)

Among the 44 HCM cases: 52% were found to carry nsSNVs, 20% carried SNVs, 13% indels frameshift variants and 1% indels non-frameshift, 12% carried nonsense changes and 2% harbored non-frameshift variants (Fig. 32).

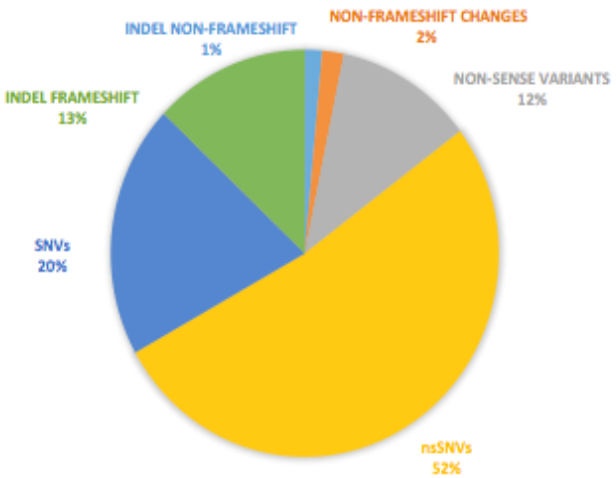


Figure 32. Proportion of the 44 patients with TTN changes (MAF ≤ 0,05) respect to the variants' category

4.2 IN SILICO PREDICTIONS

Twelve methods were employed for assessing the possible pathogenicity of the identified missense changes (Tab.10). The variants with at least 6/12 deleterious predictions have been considered possibly pathogenetic, while a "no predicted deleterious effect" was accounted for those scored with two or less deleterious predictions. The in silico scores were completely concordant for 20 variants (14 benign and 6 deleterious), but ambiguous for 19 changes which resulted in 3 to 5 deleterious predictions among 12 ones. Nine among the 14 different intronic variants were

predicted to alter a splicing site. Among the 85 different nsSNVs identifid in this study, 24 resulted in the alteration of an allele that is highly conserved across evolutionarily distant species (Tab.8). For those DNA changes, the four computational methods employed for the nucleotides' conservation analysis gave concordant scores (Tab.10).

Inferring from the type of the variant, querying of frequency and mutational databases, as well as from the output of in silico predictions, all but three (patient 3, 37 and 52) mutated cases harbored at least one non-synonymous DNA change predicted to be pathogenetic, or already reported as disease-modifying factor (Tab. 8)

Mutation	Chr	Start	Enc	Ref	Alt	SIFT	Polyphen2 HDIV	Polyphen2 HVAR	Provean	LRT	Mutation Taster	Mutation Assessor	FATHMM	RadialSVM	LRT	CADD phred	Uniprot	Domain	X-ray	Model Template	% id	Prosa Z-Score	Possible effect	HSF (altered site)	GERP++ RS	phyloP46way placental	phyloP100way vertebrate	SiPhy 29way
MYBPC3 NM_000256:c.T1664C:p.M555T	11	47363668	47363668	A	G	T (1)	B (0)	B (0)	N (4,48)	.	N (0,997)	N (-2,74)	T (-0,1)	T (-1,057)	T (0,041)	0,033	Q14896	Ig like C2-type	-	2DLT	65	-8.58	No predicted deleterious effect	.	**4,22**	1,401	**7,388**	**14,08**
RYR2 NM_001035:c.A3380G:p.E1127G	1	237730032	237730032	A	G	**D (0,25)**	**D (0,979)**	**P (0,675)**	**D (-5,34)**	**D (0)**	**D (1)**	L (1,06)	T (-0,43)	T (-0,504)	T (0,279)	**20,2**	Q92736	SPRY	-	3TOY	28	-5.23	**loss of n-anion interaction with Trp1145**	.	**5,29**	**1,995**	**7,302**	**15,238**
TMPO NM_003276:c.A1037G:p.H346R	12	98927072	98827072	A	G	T (0,42)	B (0,001)	B (0,00:)	N (0,22)	N (0,816)	N (1)	N (0,345)	T (1,7)	T (-0,979)	T (0,132)	3,347	.	.	.	.	.	.	.	.	-1,03	-0,111	-0,041	8,773
MYH6 NM_002471:c.643-5C>T	14	23873602	23873602	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**branch point**	.	.	.	.
RBM20 NM_001134363:c.G3373A:p.E1125K	10	112583294	112583294	G	A	**D (0,01)**	**P (0,911)**	B (0,293)	N (-1,80)	.	**D (0,992)**	L (1,355)	T (-0,91)	T (-0,499)	T (0,22)	**17,99**	.	.	.	.	.	.	.	.	**6,16**	**2,937**	3,214	**13,979**
MYH6 NM_002471:c.2928+5G>A	14	23862870	23862870	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**donor site**	.	.	.	.
CSRP3 NM_003476:c.T10C:p.W4R	11	19213986	19213986	A	G	T (0,38)	**P (0,888)**	**P (0,657)**	**D (-3,25)**	**D (0)**	**A (1)**	L (1,78)	T (-0,1)	T (-0,44)	T (0,244)	**25,9**	.	.	.	.	.	.	.	.	**6,02**	**2,304**	**7,415**	**16,203**
LAMA4 NM_001105206:c.A4937G:p.E1646G	6	112438986	112438986	T	C	**D (1)**	**D (1)**	**D (0,997)**	**D (-6,11)**	**D (0)**	**D (1)**	**M (2,43)**	T (-1,07)	**D (0,289)**	**D (0,582)**	**26,9**	Q16363	Laminin G-like	-	3ASI	24	-6.42	**Loss of interaction between two adjacent domains**	.	**5,6**	**2,141**	**7,17**	**15,783**
LAMA4 NM_001105206:c.G1565C:p.R522T	6	112486465	112486465	C	G	T (0,37)	B (0,324)	B (0,174)	N (-1,24)	N (0,006)	N (1)	L (1,04)	T (2,94)	T (-0,978)	T (0,008)	8,741	.	.	.	.	.	.	.	.	-1,86	-0,523	-0,169	9,422
NEBL NM_006393:c.G604A:p.G202R	10	21157673	21157673	C	T	T (0,17)	B (0,001)	B (0,00:)	N (-0,48)	N (0,002)	**D (1)**	N (0,69)	T (3,61)	T (-1,042)	T (0,025)	10,5	.	.	.	.	.	.	.	.	**4,17**	**1,541**	1,543	8,948
RYR2 NM_001035:c.9450-9C>T	1	237868504	237868504	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MYBPC3 NM_000256:c.C1112A:p.P371Q	11	47365154	47365154	G	T	**D (1)**	**D (1)**	**D (0,999)**	**D (-7,05)**	.	**D (1)**	**M (2,48)**	T (0,96)	T (-0,415)	T (0,296)	**31**	Q14896	Ig like C2-type	1PD6	.	.	.	**Possible decreased protein stability**	.	**4,99**	**2,602**	**8,974**	**18,474**
GLA NM_000169:c.A644G:p.N215S	X	100653930	100653930	T	C	**D (0,01)**	B (0,048)	B (0,004)	**D (-4,39)**	**D (0)**	**D (1)**	.	**D (-7,51)**	**D (1,044)**	**D (0,982)**	**16,95**	P06280	Melibiase	4NX5	.	.	.	**Loss of a glycosylated site**	.	**5,97**	**2,018**	**8,04**	**15,388**
DSP NM_004415:c.G137A:p.G46D	6	7542285	7542285	G	A	T (0,17)	B (0,01)	B (0,007)	N (-0,25)	N (0,867)	N (1)	N (0)	T (-0,45)	T (-1,037)	T (0,069)	6,748	.	.	.	.	.	.	.	.	0,722	-0,071	0,42	3,809
TXNRD2 NM_006440:c.G1150A:p.G384S	22	19868177	19868177	C	T	T (0,29)	B (0,367)	B (0,03)	**D (-3,01)**	**D (0,001)**	**D (1)**	**M (2,15)**	T (0,1)	T (-0,941)	T (0,116)	12,53	Q17RE6	.	.	1ZDL	84	-11.53	No predicted deleterious effect	.	**4,35**	1,411	2,985	8,752
JPH2 NM_020433:c.G1536C:p.W512C	20	42744779	42744779	C	G	T (0,18)	**D (1)**	**D (0,997)**	N (-1,95)	U (0,004)	**D (1)**	L (1,845)	T (0,1)	T (-0,508)	T (0,215)	12,45	.	.	.	.	.	.	.	.	3,12	1,441	**4,931**	**13,328**
SCN5A NM_001099405:c.A5605T:p.I1869F	3	38592204	38592204	T	A	**D (1)**	**D (0,998)**	**D (0,986)**	**D (-2,56)**	**D (0)**	**D (1)**	**M (2,005)**	**D (-3,79)**	**D (0,918)**	**D (0,892)**	**17,96**	Q14524	Interaction with FGF13	4DCK	.	.	.	**Fotential loss of interaction with FGF13**	.	**3,7**	0,887	1,661	10,421
FHL2 NM_001450:c.G109T:p.A37S	2	106002865	106002865	C	A	T (0,34)	**P (0,62)**	B (0,225)	N (-1,46)	**D (0)**	**D (0,999)**	L (0,98)	**D (-2,32)**	T (-0,375)	T (0,259)	13,95	Q14192	Zirc Finger C4-type	2MIU	.	.	Q14192	**Steric clashes with T34 and P46**	.	**4,66**	**2,811**	3,772	11,58
DSC2 NM_004949:c.C1787T:p.A596V	18	28654750	28654750	G	A	**D (0,03)**	**P (0,882)**	B (0,345)	**D (-2,85)**	N (0,006)	**D (1)**	**M (2,995)**	T (0,34)	T (-0,14)	T (0,254)	14,64	.	.	.	.	.	.	.	.	**5,27**	**2,614**	**5,918**	**16,176**
PKP2 NM_001005242:c.G76A:p.D26N	12	33049590	33049590	C	T	T (0,36)	**D (0,992)**	**P (0,874)**	N (-1,08)	N (0,004)	**D (0,999)**	L (0,895)	**D (-1,72)**	**D (0,033)**	T (0,451)	**33**	.	.	.	.	.	.	.	.	**4,07**	**2,081**	3,56	**15,41**
MYOM1 NM_003803:c.C2131T:p.R711C	18	3135623	3135623	G	A	**D (1)**	**D (1)**	**D (1)**	**D (-7,47)**	**D (0)**	**D (1)**	**H (3,89)**	T (0,15)	**D (0,396)**	**D (0,528)**	**26,8**	P52179	Fn3	.	1X5Y	40	-4.69	**Loss of inter-residues contacts**	.	**4,09**	1,498	**6,747**	**15,443**
RAF1 NM_002880:c.[G124A;C125T]:p.A42I	3	12660096	12660097	GC	AT	T (0,15)	B (0,44)	B (0,08)	N (-0,15)	**D (0)**	**D (1)**	N (0,55)	T (-0,9)	T (-0,708)	T (0,148)	**20,8**	.	.	.	.	.	.	.	.	**6,17**	**2,941**	3,851	**20,879**
TNNT2 NM_001001430:c.A252T:p.R84S	1	201334750	201334750	T	A	**D (1)**	**D (0,968)**	**P (0,712)**	**D (-2,99)**	**D (0,001)**	**D (1)**	L (1,2)	**D (-5,31)**	**D (0,887)**	**D (0,92)**	**18,22**	.	.	.	.	.	.	.	.	**3,53**	0,733	0,317	4,525
MYOM1 NM_003803:c.4485-6T>C	18	3079346	3079346	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**branch point**	.	.	.	.
RBM20 NM_001134363:c.A59G:p.D20G	10	112404271	112404271	A	G	**D (1)**	**P (0,941)**	B (0,355)	N (-0,94)	N (0,832)	N (0,884)	N (0,345)	**D (-2,93)**	T (-0,205)	**D (0,575)**	**22,2**	.	.	.	.	.	.	.	.	**4,23**	**1,54**	1,69	9,721
CAV3 NM_001234:c.C233T:p.T78M	3	8787330	8787330	C	T	T (0,15)	**D (0,973)**	**P (0,738)**	N (-0,83)	**D (0)**	**D (0,999)**	L (1,67)	**D (-3,08)**	**D (0,466)**	**D (0,71)**	**16,45**	.	.	.	.	.	.	.	.	**4,63**	**2,386**	3,58	**16,203**
DSG2 NM_001943:c.A20G:p.I70V	18	29099892	29099892	A	G	T (0,42)	B (0,001)	B (0,005)	N (-0,93)	U (0,001)	**D (0,716)**	N (0,67)	T (0,22)	T (-0,944)	T (0,11)	10,79	.	.	.	.	.	.	No predicted deleterious effect	.	**4,05**	0,919	**4,397**	10,003
DSP NM_004415:c.G5218A:p.E1740K	6	7581641	7581641	G	A	T (0,39)	**D (0,962)**	B (0,173)	N (-1,58)	**D (0)**	**D (1)**	L (1,1)	T (-0,85)	T (-0,875)	T (0,14)	11,6	.	.	.	.	.	.	.	.	**5,09**	1,496	3,14	**17,082**
MYOM1 NM_003803:c.G2132A:p.R711H	18	3135622	3135622	C	T	**D (1)**	**D (1)**	**D (1)**	**D (-4,67)**	**D (0)**	**D (1)**	**H (3,89)**	T (0,15)	**D (0,366)**	**D (0,57)**	**36**	P52179	Fn3	.	1X5Y	40	-4.67	**Loss of inter-residues contacts**	.	**5,95**	**2,821**	**7,818**	**20,381**
SCN5A NM_001099405:c.C5837T:p.S1946F	3	38591972	38591972	G	A	**D (1)**	**D (0,98)**	**P (0,641)**	**D (-2,82)**	N (0,931)	N (0,899)	N (0,345)	**D (-3,81)**	**D (0,336)**	**D (0,743)**	13,71	.	.	.	.	.	.	.	.	3,08	1,282	2,36	5,503
DSC2 NM_004949:c.C2328G:p.I776M	18	28649040	28649040	G	C	T (0,27)	B (0,008)	B (0,023)	N (-0,99)	N (0,155)	N (1)	N (-0,295)	T (0,36)	T (-1,066)	T (0,064)	8,255	.	.	.	.	.	.	.	.	-0,947	0,087	0,3	3,159
LAMA4 NM_001105206:c.T3482C:p.M1161T	6	112455744	112455744	A	G	T (0,16)	B (0)	B (0,002)	N (-1,49)	N (0,021)	**D (1)**	L (0,895)	T (-1,01)	T (-0,94)	T (0,152)	12,5	Q16363	Laminin G-like	.	2WJS	23	-6.42	No predicted deleterious effect	.	**3,53**	0,495	**5,708**	9,835

JUP NM_002230:c.909+6C>T	17	39923625	39923625	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RAF1 NM_002880:c.G1858A:p.A620T	3	12626102	12626102	C	T	**D (0,05)**	**P (0,724)**	B (0,132)	**D (-2,76)**	**D (0)**	**D (1)**	L (1,33)	T (-0,9)	T (-0,456)	T (0,286)	**15,77**	.	.	.	.	.	.	.	.	.	**5,51**	**2,868**	**4,487**	**19,61**				
SCN5A NM_000335:c.C1810G:p.L604V	3	38645283	38645283	G	C	T (1)	**D (0,992)**	**P (0,866)**	N (0,17)	N (0,461)	**D (0,565)**	N (0,66)	**D (-2,59)**	T (-0,143)	**D (0,528)**	5,963	.	.	.	.	.	.	.	.	.	3,28	**2,164**	0,885	3,132				
ABCC9 NM_005691:c.2867-5T>C	12	21998771	21998771	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MYOM1 NM_003803:c.T199G:p.S67A	18	3215023	3215023	A	C	T (1)	B (0)	B (0,001)	N (-0,13)	N (0,726)	N (1)	N (-0,345)	T (1,12)	T (-1,002)	T (0,043)	0,006	.	.	.	.	.	.	.	.	.	-0,951	-0,851	-1,373	11,115				
TNNT2 NM_001001432:c.A755T:p.K252I	1	201330414	201330414	T	A	**D (0)**	**D (0,997)**	**D (0,969)**	**D (-6,86)**	**D (0)**	**D (1)**	**M (2,565)**	**D (-3,13)**	**D (0,877)**	**D (0,851)**	**22,3**	P45379	Coiled coil	1J1D	.	.	.	No predicted deleterious effect	.	**3,73**	**1,552**	**7,699**	12,581				
SCN5A NM_000335:c.G2614A:p.D872N	3	38627355	38627355	C	T	T (0,46)	**D (1)**	**D (0,993)**	N (-0,42)	N (0,099)	N (1)	N (-0,505)	**D (-4,89)**	**D (0,308)**	**D (0,851)**	12,06	Q14524	Na-channel	.	4DXW	29	-1.93	**Loss of negative charge at Na-channel**	.	3,26	**2,169**	-0,24	8,027				
BAG3 NM_004281:c.G463A:p.A155T	10	121429645	121429645	G	A	T (0,81)	B (0)	B (0,001)	N (0,56)	N (0,061)	N (1)	N (0,205)	T (-0,74)	T (-1,06)	T (0,094)	3,627	.	.	.	.	.	.	.	.	1,69	0,515	2,03	0,918				
MYPN NM_032578:c.C3335T:p.P1112L	10	69959174	69959174	C	T	.	**D (1)**	**D (0,999)**	**D (-5,37)**	**D (0)**	**D (1)**	N (0,61)	T (-0,3)	T (-0,646)	T (0,24)	**25,9**	Q86TC9	Ig-like	.	2DM3	70	-6.29	**Possible decreased protein stability**	.	**5,38**	**2,813**	**5,719**	**14,201**				
CAV3 NM_001234:c.G221A:p.R74H	3	8787318	8787318	G	A	**D (0,01)**	**D (1)**	**D (0,99)**	**D (-4,23)**	**D (0)**	**D (1)**	**M (3,105)**	**D (-3,41)**	**D (1,032)**	**D (0,907)**	**19,29**	.	.	.	.	.	.	.	.	.	**4,63**	**2,386**	**9,181**	**16,203**				
MYH7 NM_000257:c.G676A:p.A226T	14	23900850	23900850	C	T	T (0,21)	**D (0,989)**	**D (0,948)**	N (-1,82)	.	**D (1)**	**M (3,24)**	**D (-3,67)**	**D (0,932)**	**D (0,906)**	**20,1**	P12883	Myosin head	4P7H	.	.	.	**Clashes with residues A335-F336**	.	3,28	**1,84**	1,119	11,973				
DSP NM_004415:c.G6188A:p.R2063Q	6	7583683	7583683	G	A	T (0,54)	**D (0,998)**	**P (0,872)**	N (-1,91)	**D (0)**	N (0,639)	L (1,245)	T (-0,73)	T (-0,751)	T (0,267)	**19,86**	.	.	.	.	.	.	.	.	.	**4,98**	**2,459**	3,613	**18,609**				
DSC2 NM_004949:c.C595T:p.R199C	18	28669437	28669437	G	A	**D (0)**	**P (0,862)**	B (0,266)	**D (-4,68)**	N (0,451)	N (1)	**M (3,2)**	T (0,56)	T (-0,589)	T (0,247)	10,26	Q02487	Cadherin	.	3K5R	39	-6.36	**Possible disulfide bond with C197**	.	-1,58	-0,645	-0,446	**14,629**				
MYH7 NM_000257:c.G3346A:p.E1116K	14	23889434	23889434	C	T	**D (0,04)**	**D (1)**	**D (0,999)**	**D (-2,69)**	.	**D (1)**	**M (3,07)**	**D (-1,71)**	**D (0,693)**	**D (0,773)**	**26,6**	.	.	.	.	.	.	.	.	.	**5,13**	**2,678**	**7,521**	**18,771**				
MYH6 NM_002471:c.G622A:p.D208N	14	23873940	23873940	C	T	T (0,1)	B (0,006)	B (0,063)	N (-1,25)	.	**D (0,967)**	N (0,47)	T (-0,57)	T (-0,796)	T (0,152)	**16,84**	.	.	.	.	.	.	.	.	.	3,42	**1,909**	2,163	**13,909**				
MYH6 NM_002471:c.G4193A:p.R1398Q	14	23857530	23857530	C	T	**D (0,01)**	B (0,222)	B (0,076)	**D (-2,81)**	.	**D (1)**	**M (3,425)**	T (-1,27)	**D (0,174)**	**D (0,54)**	**15**	.	.	.	.	.	.	.	.	.	**4,64**	**2,292**	**7,528**	**17,892**				
DSG2 NM_001943:c.G2033C:p.G678A	18	29122514	29122514	G	C	T (1)	B (0,001)	B (0,001)	N (-0,45)	N (0,928)	N (1)	N (-0,805)	T (0,4)	T (-1,015)	T (0,04)	5,823	.	.	.	.	.	.	.	.	.	0,162	-0,176	-0,487	0,709				
PKP2 NM_001005242:c.C1445T:p.T482M	12	32994073	32994073	G	A	T (0,12)	B (0,198)	B (0,032)	N (-1,40)	N (0,002)	N (1)	L (1,56)	T (-1,07)	T (-0,835)	T (0,225)	3,327	Q99959	.	3TT9	.	.	.	No predicted deleterious effect	.	2,01	0,068	0,502	8,816				
MYH7 NM_000257:c.G428A:p.R143Q	14	23901922	23901922	C	T	**D (0,04)**	**D (0,998)**	**P (0,888)**	N (-2,39)	.	**D (1)**	L (1,65)	**D (-2,28)**	**D (0,494)**	**D (0,691)**	**32**	P12883	Myosin head	4P7H	.	.	.	**Loss of a salt-bridge with Asp159**	.	**3,61**	**2,015**	**4,65**	**15,807**				
MYBPC3 NM_000256:c.A649G:p.S217G	11	47371330	47371330	T	C	**D (0,01)**	**P (0,907)**	**P (0,605)**	**D (-2,69)**	.	**D (0,905)**	L (1,59)	T (-0,43)	T (-0,445)	T (0,28)	13,31	.	.	.	.	.	.	.	.	.	**3,67**	0,801	**4,944**	10,479				
MYH7 NM_000257:c.C2167T:p.R723C	14	23895023	23895023	G	A	T (0,25)	**D (1)**	**D (0,995)**	**D (-4,82)**	.	**D (1)**	**M (2,06)**	**D (-2,34)**	**D (0,39)**	**D (0,732)**	**19,53**	P12883	Myosin head	4P7H	.	.	.	**Loss of interaction between two adjacent domains**	.	**3,94**	**2,531**	1,866	**13,979**				
TMPO NM_003276:c.G1696A:p.D566N	12	98927731	98927731	G	A	**D (0)**	B (0,079)	B (0,088)	N (-0,75)	N (0,002)	**D (1)**	N (0,695)	T (-0,01)	T (-0,875)	T (0,157)	**15,84**	P42166	Four-stranded coiled coil	.	2V0X	79	-5.07	No predicted deleterious effect	.	**5,08**	**1,539**	3,006	10,994				
MYL2 NM_000432:c.G34T:p.G12C	12	111356967	111356967	C	A	T (0,08)	**D (1)**	**D (0,996)**	**D (-2,86)**	**D (0)**	**D (1)**	L (0,895)	T (-0,96)	**D (0,122)**	**D (0,553)**	14,45	.	.	.	.	.	.	.	.	.	**5,33**	**2,491**	**7,197**	**17,805**				
VCL NM_003373:c.A1907G:p.H636R	10	75860740	75860740	A	G	**D (0,04)**	**D (0,989)**	**D (0,954)**	**D (-3,09)**	**D (0)**	**D (1)**	**M (2,005)**	T (0,59)	T (-0,43)	T (0,309)	**21,6**	P18206	N-terminal globular head	.	1ST6	95	-13.63	**Possible misfolding of four-helix bundle**	.	**5,04**	**2,016**	**8,659**	**15,063**				
ACTN2 NM_001103:c.C1484T:p.T495M	1	236911044	236911044	C	T	T (0,06)	**D (1)**	**D (1)**	N (-2,14)	**D (0)**	**D (1)**	L (1,3)	T (0,66)	T (-0,703)	T (0,252)	**23,5**	P35609	Spectrin	1HCI	.	.	.	No predicted deleterious effect	.	**5,73**	**2,854**	**5,842**	**20,27**				
SCN5A NM_001099405:c.C2074A:p.Q692K	3	38639408	38639408	G	T	**D (0,04)**	B (0)	B (0,002)	N (-1,11)	N (0,372)	N (0,958)	L (1,245)	**D (-3,68)**	**D (0,062)**	**D (0,746)**	10,67	.	.	.	.	.	.	.	.	.	1,95	0,211	-0,035	11,668				
DMD NM_004017:c.G1646A:p.R549Q	X	31164440	31164440	C	T	T (0,36)	**D (0,999)**	**D (0,986)**	N (0,10)	U (0,006)	**D (1)**	N (0,2)	T (3,89)	T (-0,654)	T (0,261)	**28,5**	.	.	.	.	.	.	.	.	.	**5,53**	**2,553**	**6,826**	**18,451**				
DMD NM_004011:c.C3164T:p.T1055I	X	31854848	31854848	G	A	T (0,32)	B (0,235)	B (0,144)	N (-0,73)	U (0,003)	**D (1)**	N (0,695)	T (1,33)	T (-1,081)	T (0,065)	10,65	.	.	.	.	.	.	.	.	.	**4,72**	**2,36**	3,932	**13,433**				
LAMP2 NM_001122606:c.C418G:p.L140V	X	119582963	119582963	G	C	T (0,8)	B (0,012)	B (0,022)	N (0,25)	N (0,04)	N (1)	N (-0,05)	T (1,45)	T (-1,031)	T (0,027)	1,336	.	.	.	.	.	.	.	.	.	.	-6,74	-1,237	-4,427	1,415				
DSG2 NM_001943:c.G2147A:p.G716E	18	29122628	29122628	G	A	T (0,98)	B (0,132)	B (0,06)	N (-1,68)	N (0,043)	N (1)	N (0,55)	T (0,28)	T (-1,012)	T (0,094)	11,87	.	.	.	.	.	.	.	.	.	.	**4,14**	0,789	1,617	9,789				
LAMA4 NM_001105206:c.4665+8G>T	6	112441478	112441478	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**IIE site**	.	.	.	.				
MYBPC3 NM_000256:c.2309-2A>G	11	47359347	47359347	T	C	.	.	.	.	.	**D (1)**	.	.	.	.	**16,83**	.	.	.	.	.	.	.	.	**acceptor site**	**4,66**	1,961	**7,501**	**13,428**				
RYR2 NM_001035:c.G1454A:p.R485Q	1	237617852	237617852	G	A	T (0,07)	**D (0,997)**	**P (0,821)**	**D (-2,94)**	U (0)	**D (1)**	**M (2,575)**	**D (-3,96)**	**D (1,018)**	**D (0,925)**	**27,2**	Q92736	RYDR_ITPR	.	4JKQ	93	-9.43	**Loss of π-cation interaction with Tyr462**	.	**5,62**	**2,645**	**9,804**	**19,666**				
ABCC9 NM_005691:c.G2200A:p.V734I	12	22017410	22017410	C	T	T (0,45)	B (0,004)	B (0,009)	N (-0,10)	N (0,044)	**D (1)**	N (0,75)	**D (-3,27)**	T (-0,808)	**D (0,642)**	13,11	O60706	ABC_tran	.	2CBZ	36	-8.25	No predicted deleterious effect	.	**4,97**	**2,575**	2,757	**18,028**				
CALR3 NM_145046:c.A1036G:p.I346V	19	16590061	16590061	T	C	T (0,29)	**D (0,983)**	**P (0,672)**	N (0,10)	N (0,042)	N (0,999)	N (0,63)	T (0,99)	T (-1,063)	T (0,085)	11,06	Q96L12	PB035848	.	3POS	61	-1.93*	No predicted deleterious effect	.	2,67	0,28	1,313	6,573				
TNNT2 NM_001001430:c.264+7G>A	1	201334731	201334731	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**donor site**	.	.	.	.				
MYH7 NM_000257:c.G4402A:p.E1468K	14	23886479	23886479	C	T	**D (0)**	**D (0,999)**	**D (0,999)**	**D (-2,94)**	.	**D (1)**	**M (3,495)**	T (-1,48)	**D (0,727)**	**D (0,768)**	**36**	.	.	.	.	.	.	.	.	.	**5,37**	**2,793**	**7,314**	**19,297**				
ILK NM_001014795:c.G1086C:p.Q362H	11	6631386	6631386	G	C	**D (0,01)**	**D (0,998)**	**D (0,929)**	**D (-3,99)**	**D (0)**	**D (1)**	**M (2,83)**	**D (-1,68)**	**D (0,434)**	**D (0,693)**	**15**	Q13418	Pkinase_tyr	3REP	.	.	.	No predicted deleterious effect	.	3,17	0,795	3,198	10,671				
LAMA4 NM_001105206:c.G1967A:p.S656N	6	112476142	112476142	C	T	T (0,34)	B (0,001)	B (0,002)	N (-0,54)	N (0,1)	**D (1)**	L (0,895)	T (2,59)	T (-0,995)	T (0,009)	9,958	.	.	.	.	.	.	.	.	.	3,39	0,749	0,577	4,097				
MYH6 NM_002471:c.G68A:p.R23H	14	23876365	23876365	C	T	**D (0,05)**	**D (0,998)**	**P (0,813)**	**D (-2,81)**	.	**D (1)**	**M (2,71)**	**D (-2,05)**	**D (0,596)**	**D (0,714)**	**17,94**	P13533	Myosin rotor	4DB1	.	.	.	**Loss of a salt-bridge with E20**	.	**3,53**	**1,974**	**7,464**	**15,232**				
TPM1 NM_001018008:c.C134T:p.A45V	15	63349185	63349185	C	T	**D (0)**	**D (1)**	**D (0,993)**	**D (-3,35)**	N (0,004)	**D (1)**	**H (3,855)**	T (-1,28)	**D (0,843)**	**D (0,793)**	**36**	.	.	.	.	.	.	.	.	.	**5,57**	**2,628**	**7,818**	**18,541**				
PKP2 NM_001005242:c.C2299A:p.R767S	12	32949101	32949101	G	T	**D (0,01)**	B (0,434)	B (0,219)	**D (-3,22)**	N (0,004)	**D (1)**	**M (2,585)**	T (0,78)	T (-0,636)	T (0,201)	**17,8**	Q99959	Arm repeat 8	.	1XM9	36	-4.71	**Loss of salt-bridge with E852**	.	**5,06**	**2,508**	3,303	**17,583**				
DSP NM_004415:c.G88A:p.V30M	6	7542236	7542236	G	A	T (1)	B (0)	B (0)	N (0,40)	N (0,68)	**A (0)**	N (-0,695)	T (-0,46)	T (-1,128)	T (0,028)	0,004	.	.	.	.	.	.	.	.	.	-7,34	-3,024	-0,688	4,332				
RYR2 NM_001035:c.A4273G:p.T1425A	1	237755151	237755151	A	G	T (0,28)	B (0,01)	B (0,031)	**D (-2,89)**	N (0,003)	**D (1)**	L (1,7)	T (-0,32)	T (-0,759)	T (0,247)	**15**	.	.	.	.	.	.	.	.	.	**5,32**	**2,225**	**6,401**	**15,443**				
MYBPC3 NM_000256:c.821+3G>A	11	47369405	47369405	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**IIE site**	.	.	.	.				
ABCC9 NM_005691:c.C3460T:p.R1154W	12	21995261	21995261	G	A	**D (0,01)**	**D (1)**	**D (0,981)**	**D (-3,79)**	**D (0)**	**D (1)**	**H (3,535)**	**D (-2,56)**	**D (0,913)**	**D (0,855)**	**17,03**	.	.	.	.	.	.	.	.	.	**4,1**	1,312	2,824	12,816				
TXNRD2 NM_006440:c.375-8C>T	22	19905748	19905748	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				
DSP NM_004415:c.C3956T:p.T1319I	6	7580379	7580379	C	T	T (0,26)	**D (0,998)**	**D (0,987)**	N (-1,03)	**D (0)**	**D (1)**	L (1,1)	**D (-2,86)**	**D (0,392)**	**D (0,725)**	**15,02**	.	.	.	.	.	.	.	.	.	**5,27**	**2,495**	**4,343**	**18,553**				
ABCC9 NM_005691:c.2238-1G>A	12	22015989	22015989	C	T	.	.	.	.	.	**D (1)**	.	.	.	.	**24,1**	.	.	.	.	.	.	.	.	**acceptor site**	**5,44**	**2,723**	**4,703**	**18,618**				
DTNA NM_001198938:c.C2095T:p.R699C	6	7580379	7580379	C	T	.	.	.	N (-1,03)	.	.	.	.	.	.	**15,02**	.	.	.	.	.	.	.	.	.	.	.	.	.				
LMNA NM_001257374:c.1363-7T>C	1	156108272	156108272	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				
ABCC9 NM_005691:c.816+11G>A	12	22068591	22068591	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**donor site**	.	.	.	.				
JPH2 NM_020433:c.G1513A:p.G505S	20	42744802	42744802	C	T	T (0,71)	B (0,019)	B (0,002)	N (0,13)	U (0,122)	N (1)	L (1,39)	T (0,26)	T (-1,061)	T (0,039)	1,421	.	.	.	.	.	.	.	.	.	.	1,03	-0,002	0,214	3,534				

Table 10. Results of in silico prediction of the non-synonimus changes found in 41 HCM patients and in 3-HCM-related cases by 6. Deleterious predictions are in bold (Bottillo *et al.;* 2016b)

* Quality of the modeled comparable to an NMR structure, given its sequence identity and length

Chr: chromosome; **Ref**: reference nucleotide; **Var**: variant nucleotide; **X-ray**: PDB Code of the experimentally determined protein structure, when available; **Model Template**: PDB Code of the experimentally determined nearest homologous protein structure; **% id**: % identity between target sequence to be modeled and nearest homologous protein structure (template); **Prosa Z-score**: overall model quality according to the Prosa validation too; **IIE**: intron identity element

SIFT, FATHMM and RadialSVM predictions: **D**: Deleterious; **T**: Tolerated

Polyphen2 predictions: **D**: Probably damaging; **P**: possibly damaging; **B**: benign

Provean predictions: **D**: Deleterious; **N**: Neutral

LRT predictions: **D**: Deleterious; **N**: Neutral; **U**: Unknown

Mutation Taster predictions: **A**: disease_causing_automatic; **D**: disease_causing; **N**: polymorphism; **P**: polymorphism_automatic

Mutation Assessor predictions: **H**: high; **M**: medium; **L**: low; **N**: neutral. H/M means functional and L/N means non-functional

CADD_phred predictions: a cutoff was put at scaled C-score=15. A scaled C-score of greater of equal 15, indicates that the variant is predicted to be one of the ~3% most deleterious substitutions in the human genome

GERP++, PhyloP and SiPhy predictions: higher scores are more deleterious

Regarding the molecular modeling (in collaboration with Prof. Paiardini - Dept. of Biochemical Sciences “A. Rossi Fanelli”, Sapienza University of Rome, Rome, Italy), protein structures were experimentally determined by X-ray crystallography, or were inferred by homology modeling means (i.e., availability of a structural template with percentage of identity 4 20%). Protein models were built using the homology mod- eling approach implemented in modeler-9 package (Eswar *et al.;* 2007) (Bottillo *et al.;* 2016b). PSI-BLAST was used to find suitable structural templates for each sequence to model (Friedberg *et al.;* 2000) (Bottillo *et al.;* 2016b). The sequences of each protein target to model and its structural template were then aligned by using the program CLUSTALW (Thompson *et al.;* 1994) (Bottillo *et al.;* 2016b) and manually manipulated to optimize the matching of several characteristics, including the observed and predicted secondary structural elements, the hydrophobic regions in the three-dimensional structures, the structurally and functionally conserved residues, and indel regions in the structures (Bottillo *et al.;* 2016b). Then, ten different models were built for each target protein and evaluated using several criteria. The model displaying the lowest objective function (Burke *et al.;* 1999) (Bottillo *et al.;* 2016b), which measures the extent of violation of constraints from the structural templates, was taken as the representative model. Superimposition and root-mean-square deviation (RMSD) calculation of Cα traces of the 10 models were performed to detect the most variable and therefore less reliable modeled regions. These invariably corresponded to loop elements. Procheckwas used to monitor the stereochemical quality of the representative models (Laskowski *et al.;* 1996) (Bottillo *et al.;* 2016b), whereas ProsaIIwas used to measure the overall protein quality in packing and solvent exposure (Sippl *et al.;* 1993) (Bottillo *et al.;* 2016b). Mutations on protein structures were carried out using the “Mutate model” script implemented in modeler-9 package (Eswar *et al.;* 2007) (Bottillo *et al.;* 2016b).The script takes as input a given three-dimensional structure of a protein (experimentally determined or predicted), and mutates a single residue. The residue sidechain's position is then optimized by energy minimization and refined by molecular dynamics simulations. Prediction of protein stability upon mutation was carried out using the DUET server (Pires *et al.;* 2014) (Bottillo *et al.;* 2016b). Sequence identity between the modeled domain and its closest template ranged from 23% (Laminin G-like domain of LAMA4), to nearly 95% (N-terminal globular head domain of VCL). However, in spite of the low value of sequence identity measured in some cases, all of the models resulted in a good overall quality (Prosa Z-score < -2.00), except for CALR3 and SCN5. Given the short length of the predicted PB035848 domain of CALR3 (residues 294-347) and its sequence identity with its template (61%), the measured Prosa Z-score (-1.93) nonetheless indicated a model of quality comparable to a Nuclear Magnetic Resonance (NMR) structure (Fig.33-34) (Sippl *et al.;* 1993) (Bottillo *et al.;* 2016b).

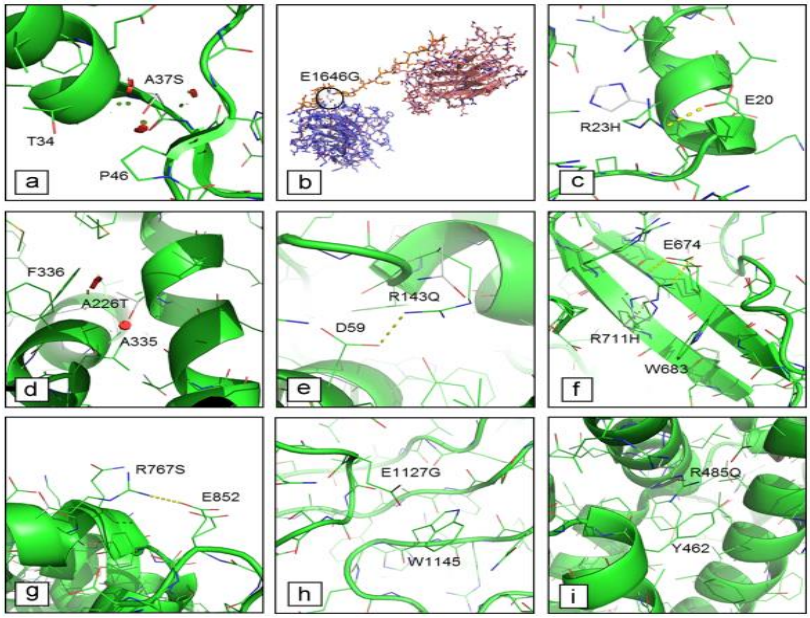


Figure 33. Structural comparison of wild-type and mutant forms for (a) FLH2 A37S; (b) LAMA4 E1646G; (c) MYH6 R23H; (d) MYH7 A226T; (e) MYH7 R143Q; (f) MYOM1 R711H; (g) PKP2 R767S; (h) RYR2 E1127G; (i) RYR2 R485Q. The mutation is indicated in white. The predicted structural effects of mutations are: (a, d) steric hindrance (red circles); (b) local misfolding of linker domain (orange); (c, e, f, g) loss of important inter-residues contacts; (h) loss of a **π**-anion interaction; (i) loss of a **π**-cation interaction (Bottillo *et al.;* 2016b)

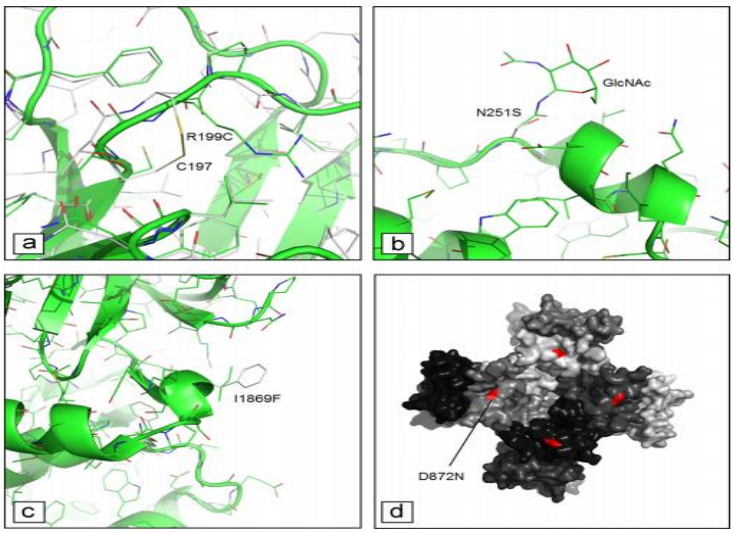


Figure 34. Effects of nsSNVs for: (a) the cadherin domain of DSC2. The mutant R199C in the cadherin domain of DSC2 is predicted to introduce a disulfide bond with the near Cys197 residue (Cα-Cαdistance ≅6Âe), and possibly to result in local misfolding of the cadherin domain; (b) the melibiase domain of GLA. Mutant N215S of the melibiase domain of GLA results in the loss of a glycosylated site probably affecting the protein structure and/or function; (c) the FGF13 interaction domain of SCN5. Mutation I869F localizes on a solvent-exposed hydrophobic path of the domain of interaction with fibroblast growth factor 13 (FGF13). The I869F mutation could affect the recognition of the FGF13 protein; (d) the Na-Channel of SCN5. The mutant D872N results in the loss of a negative charge that is approximately located at the Na-channel domain of SCN5, probably affecting cations conductance of the channel. The approximate position of the negatively charged Asp872 residue is shown in red, in each of the four protein subunits forming the channel (Bottillo *et al.;* 2016b)

4.3 GENOTYPE–PHENOTYPE CORRELATIONS

Genotype phenotype correlation between nsSNVs identified in the 62 cardiovascular genes and the 41 patients with non-syndromic hypertrophic cardiomyopathy was carried out on this study (Tab.11). It was observed an inverse correlation between the age at diagnosis and the total number of nsSNVs: the mean age at diagnosis was indeed 60 years in the group without variants and 43.8 years in the group with variants (P = 0.01). The presence of nsSNVS in the genes involved in intracellular Ca++ homeostasis was significantly correlated to an earlier HCM onset: the mean age at diagnosis was in fact 35.8 years between the cases carrying those variants, versus 48.2 years in the cases without them (P = 0.03). The mean value of maximum wall thickness was lower both in the group carrying nsSNVs in genes for K+ and Na+ channels (P = 0.03), and in the group not harboring variants for Ca++ homeostasis (P = 0.05). Moreover it was observed a correlation between maximum wall thickness and the family history, as the mean of the MWT value of was lower in the sporadic group (18.055 mm) than in the familial group (22.3 mm) (P = 0.05). The occurrence of non-synonymous changes in cytoskeletal loci was correlated with the presence of non-sustained ventricular tachycardia: NSTV was indeed present in 40% of the patientscarrying cytoskeletal nsSNVs, versus 11% of patients not carrying them (P = 0.05). NSTV was also correlated with familial cases, respect to sporadic ones (P = 0.02). Cytoskeletal variants were correlated with a minor grade (grade 0–1) of diastolic dysfunction (P = 0.004) and, regarding New York Heart Association (NYHA) functional classification, sarcomeric variants were present in all grade I (no limitation of physical activity), in 56% of grade II (slight limitation of physical activity), and in 33% of grade III (marked limitation of physical activity) patients (P = 0.05). The prevalence of male sex was higher, respect to female in the group carrying at least one nsSNV compared with the HCM patients with no rare nsSNV (P = 0.01).Finally we identified a statistically significant correlation between the presence of an implantable cardioverter-defibrillator and familyhistory (P = 0.03): the implantation of ICD resulted to be linked to a familial framework.

Pt	Sex	Dgn Age	Fam His	HTX	NYHA	MWT	LVM	CMRI	LVEF	Familial SCD	HOCM	SAR	Ablation	NSTV	Sync/Presync	VO_2	Diast Dys	Failure	Thor pn	ECG Hyp	Cond Abn	Hpt	ICD	PMK	Myct	CVG	AMI/NSTEMI	CAB	Variants	Sarc	Des	K^+-Na^+	Spl/E/N	Cyt	Z-disk	Ca^{++} hmst	Other
1	M	36	F	-	2	29	91	DI	53	0	+	-	-	-	-	75	2	-	+	-	-	-	-	-	-	n.e.	-	-	2	1	-	-	-	-	-	1	-
2	F	63	F	-	3	24	n.e.	n.e.	45	n.e.	+	AF	-	-	-	46	2	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
3	M	64	F	-	2	18	90	-	65	0	-	-	-	-	-	n.e.	2	-	-	+	-	+	-	-	-	n.e.	-	-	1	-	-	-	1	-	-	-	-
4	F	42	F	-	2	18	90	MI	65	0	-	-	-	+	+	55	2	-	+	-	-	-	+	-	-	n.e.	-	-	-	-	-	-	-	-	-	-	-
5	M	63	S	-	2	27	137	-	60	0	-	-	-	-	-	n.e.	2	-	+	+	-	+	-	-	-	n.e.	-	-	1	1	-	-	-	-	-	-	-
6	M	60	F	-	2	20	105	-	65	0	+	AF	-	-	-	77	2	-	+	-	LBBB	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-
7	F	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	-	-	-	-	-	-	-	-	-
8	M	n.e.	S	-	1	16	98	-	65	0	+	PSVT	-	+	-	48	2	-	-	+	-	-	-	-	-	-	-	-	5	1	-	-	1	2	1	-	-
9	F	40	F	-	2	17	75	-	65	1	-	AF	-	-	-	65	2	-	-	+	-	+	-	+	-	n.e.	-	-	3	2	-	-	-	-	-	1	-
11	M	36	F	#	3	22	133	MI	50	0	-	AF	-	+	-	41	3	+	-	-	-	-	+	-	-	-	-	-	4	-	2	1	-	-	-	1	-
12	F	65	S	-	2	16	n.e.	n.e.	67	0	+	-	-	-	-	n.e.	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	M	46	F	-	2	24	105	MI	70	0	+	AF	-	-	-	87	2	-	-	+	-	-	-	-	-	n.e.	-	-	-	-	-	-	-	-	-	-	-
14	F	54	S	-	2	18	n.e.	n.e.	65	0	+	-	-	-	-	70	2	-	+	+	-	+	-	-	-	n.e.	-	-	1	-	1	-	-	-	-	-	-
15	M	36	S	-	1	15	101	-	80	0	-	-	-	-	-	84	-	-	+	+	-	+	-	-	n.e.	n.e.	n.e.	n.e.	3	1	-	-	2	-	-	-	-
16	F	60	S	-	2	19	n.e.	n.e.	60	0	-	-	-	+	-	n.e.	2	-	-	+	-	-	-	-	-	n.e.	-	-	-	-	-	-	-	-	-	-	-
17	F	50	S	-	2	14	91	MI	52	0	-	-	-	-	-	78	3	+	+	+	LBBB	-	-	-	-	n.e.	-	-	4	2	-	1	1	-	-	-	-
18	M	36	S	#	3	16	100	MI	57	1	-	PSVT	-	-	-	60	3	+	+	-	-	-	-	-	-	n.e.	-	-	2	-	2	-	-	-	-	-	-
19	M	36	S	+	3	14	n.e.	n.e.	50	0	-	PSVT	-	-	-	65	3	+	-	-	LAFB	-	-	-	-	n.e.	-	-	6	1	2	1	-	1	-	-	1
20	F	19	F	-	2	28	112	MI	71	0	-	PSVT	+	+	-	87	-	-	-	+	-	-	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	2	-	1	-	-	1	-	-	-
21	M	22	S	-	2	30	148	DI	75	0	+	-	-	-	-	60	2	-	-	+	RBBB	-	+	-	-	n.e.	-	-	5	2	-	1	-	-	-	2	-
22	M	46	s	-	1	27	92	T	75	0	+	-	-	-	-	70	2	-	-	+	-	+	-	-	-	n.e.	-	-	2	1	-	-	-	-	-	-	1
23	M	18	F	-	1	27	151	T	75	0	-	-	-	+	+	53	-	-	+	+	-	-	+	-	-	n.e.	-	-	4	2	1	-	-	1	-	-	-
25	F	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	4	1	-	-	1	-	1	-	1
26	F	59	F	-	2	19	75	MI	65	1	-	-	-	+	-	n.e.	1	-	-	-	-	-	-	-	-	-	-	-	5	1	1	2	-	1	-	-	-
29	F	50	F	+	3	13	n.e.	n.e.	25	0	-	-	-	-	-	40	3	+	-	-	-	-	+	-	-	-	-	-	1	-	-	1	-	-	-	-	-
31	M	45	F	-	2	26	n.e.	n.e.	70	0	+	PSVT	+	-	-	n.e.	2	-	-	+	+	-	-	-	+	n.e.	-	-	3	1	1	-	-	-	-	1	-
32	M	48	n.e.	-	2	17	90	-	70	0	+	-	-	-	-	85	2	-	-	+	-	+	-	-	-	n.e.	-	-	3	-	1	1	1	-	-	-	-
33	M	55	S	-	2	22	94	-	67	0	+	-	-	-	-	70	2	-	-	-	-	+	-	-	-	-	-	-	2	1	-	-	-	1	-	-	-
34	M	60	S	-	1	18	90	-	73	0	-	PSVT	-	-	-	60	1	-	-	+	-	+	-	-	-	n.e.	-	-	6	2	1	2	-	-	-	-	1
35	M	40	S	-	2	16	87	MI	74	0	-	PSVT	-	-	+	60	2	-	+	+	-	-	-	-	-	n.e.	n.e.	n.e.	2	1	-	1	-	-	-	-	-
36	F	28	S	-	2	17	70	MI	65	0	+	PSVT	-	-	-	60	2	-	-	+	-	-	-	-	-	n.e.	-	-	1	1	-	-	-	-	-	-	-
37	M	60	S	-	2	22	95	MI	70	0	+	-	-	-	-	62	2	-	-	+	RBBB+LAFB+	-	-	-	-	n.e.	-	-	2	-	2	-	-	-	-	-	-
38	F	55	F	-	2	18	85	-	80	0	+	-	-	+	-	60	2	-	-	-	-	-	-	-	-	n.e.	-	-	2	1	-	1	-	-	-	-	-
43	M	16	F	+	3	25	200	DI	45	1	-	-	-	+	+	27	3	+	-	+	RBBB+LAFB-	+	-	-	-	n.e.	-	n.e.	2	2	-	-	-	-	-	-	-
44	M	20	F	-	2	26	n.e.	n.e.	70	0	+	-	-	-	-	52	2	-	+	+	-	-	-	-	n.e.	n.e.	n.e.	n.e.	4	2	2	-	-	-	-	-	-
45	M	n.e.	F	-	2	30	112	DI	50	0	-	-	-	+	+	45	3	-	+	+	-	-	+	-	n.e.	n.e.	n.e.	n.e.	2	2	-	-	-	-	-	-	-
46	M	60	F	-	1	18	91	MI	68	0	-	PSVT	-	-	-	90	2	-	-	-	RBBB+LAFB+	-	-	-	-	-	+	n.e.	1	1	-	-	-	-	-	-	-
49	F	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	4	1	1	-	2	-	-	-	-
51	F	58	S	-	2	17	n.e.	n.e.	60	0	+	SA	-	-	+	65	2	-	-	-	-	-	n.e.	+	n.e.	n.e.	n.e.	n.e.	2	1	-	-	-	-	1	-	-
52	F	58	S	-	2	1	87	MI	80	0	-	-	-	-	+	70	1	-	-	-	-	-	-	+	-	n.e.	n.e.	n.e.	4	-	-	1	-	2	1	-	-
55	F	70	n.e.	n.e.	n.e.	17	81	-	80	n.e.	-	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	-	-	-	-	-	-	-	-	-
Syndromic HCM																																					
10-Fabry	F	60	S	-	2	14	104	-	77	0	-	-	-	-	-	60	2	-	-	+	-	-	-	-	-	n.e.	-	-	4	1	1	-	-	-	-	-	2
24-Cantù	F	7	S	-	n.e.	n.e.	n.e.	n.e.	n.e.	0	-	-	-	-	n.e.	-	-	-	+	-	-	-	-	-	-	-	-	-	5	1	2	1	-	-	-	1	-
27-Danon	F	14	F	+	3	32	220	MI	55	0	-	TPSV	+	-	+	65	3	+	-	+	LBBB	-	+	-	-	-	-	-	2	-	-	-	1	-	-	-	1

Table 11. Clinical and molecular features of the 44 HCM patients. Patients 2, 4, 6, 7, 12, 13, 16 and 55 (highlighted in grey) did not harbor any low-frequency nsSNVs in the analyzed genes. **Pt:** patient; **Dgn Age:** age at diagnosis (years); **n.e.:** not evaluated; **+:** present; **-:** absent; **Fam His:** Family History (**F:** Familial; **S:** Sporadic); **HTX:** Heart Transplantation; **#:** dead before HTX; **NYHA:** New York Heart Association functional class; **MWT:** Maximal Wall Thickness (mm); **LVM:** Left Ventricular Mass (at CMRI) (mg/m^2); **CMRI:** cardiac magnetic resonance areas of contrast (**DI:** diffused intramyocardial; **MI:** mild intramyocardial; **T:** transmural); **LVEF:** Left Ventricular Ejection Fraction (%); **SCD:** Sudden Cardiac Death (**0:** no relatives with SCD; **1:** one or more relatives with SCD); **HOCM:** Obstructive Cardiomyopathy (outflow obstruction); **SAR:** Supraventricular Arrhythmias (**PSVT:** Paroxysmal Supraventricular Tachycardia; **AF:** Atrial Fibrillation; **SA:** Sinus Arrest); **NSTV:** Non-Sustained Ventricular Tachycardia; **Sync/Presync:** Syncope/Presyncope; **VO_2:** maximal oxygen uptake (%); **Diast Dys:** Diastolic Dysfunction (grade); **Thor pn:** thoracic pains; **ECG Hyp:** ElectroCardioGram Hypertrophy criteria; **Cond Abn:** Conduction abnormalities (**RBBB:** Right Bundle Branch Block; **LBBB:** Left Bundle Branch Block; **LAFB:** Left Anterior Fascicular Block); **Hpt:** Hypertension; **ICD:** Intracardiac Defibrillator; **PMK:** Pacemaker; **Myct:** Myectomy; **CVG:** Coronary-Ventriculography; **AMI/NSTEMI:** Acute Myocardial Infarction Non-ST elevation Myocardial Infarction; **CAB:** Coronary Bypass; **Variants:** totalnumber of nsSNVs; **Sarc:** number of sarcomeric nsSNVs; **Des**: number desmosomal nsSNVs; **K^+-Na^+:** number of nsSNVs in genes for K+ and Na^+ channels and interacting proteins; **Spl/E/N:** number of nsSNVs in mRNA splicing, cellular enzymes and nuclear genes; **Cyt:** number of cytoskeletal nsSNVs variants; **Z-disk:** number of Z-disk nsSNVs; **Ca^{++} hmst:** number of intracellular calcium homeostasis nsSNVs (Bottillo *et al.;* 2016a)

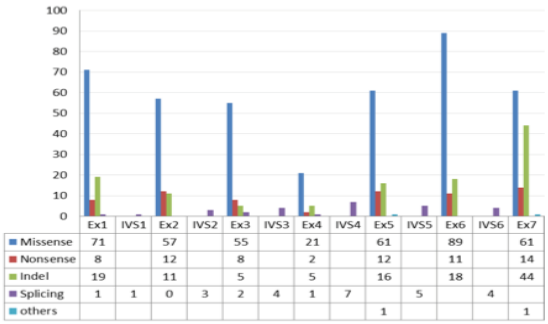
4.4 PATIENTS WITH HCM ASSOCIATED WITH A SYNDROMIC PHENOTYPE

Among the syndromic patients, case 10 was found to carry the p.N215S mutation in the *GLA* gene, previously associated with a mild form of Fabry disease (Davies *et al.;* 1993; Eng *et al.;* 1993), case 24 harbored the *ABCC9* p.R1154W mutation causing Cantù's syndrome (Harakalova *et al.;* 2012; van Bon *et al.;* 2012) and case 27 carried the novel *LAMP2* p.F151fs mutation (Bottillo *et al.;* 2016c). In addition, they were found to carry other rare nsSNVs (Tab.9).

4.4.1 Patient 10

Case 10 was a 65 years old woman affected by a mild form of Fabry's disease. Genetic testing was performed by next generation cardio panel including 62 genes and the patient was found to carry the *GLA* c.A644G,p.N215S mutation. This mutation was already characterized like pathogenetic and it causes Fabry disease (Davies *et al.;* 1993) (Eng *et al.;* 1993).

In order to determine an association between this mutation and the specific cardiac phenotype of this patient, we made an exhaustive literature review (http://fabry-database.org/),figure 35 shows the general distribution of the known *GLA* mutation correlated to the pathogenesis of Fabry's disease (Fig.35). Over 500 pathologic *GLA* allelic variants have been identified, including missense and nonsense mutations, large and small gene rearrangements, and splicing defects. Most mutations are family-specific, occurring only in single pedigrees. However, mutations at CpG dinucleotides have been identified in unrelated families of different ethnic or geographic backgrounds. Haplotype analysis of mutant alleles that occur in two or more families revealed that individuals with rare alleles are most likely related, whereas individuals with mutations involving CpG dinucleotide "hot spots" are not. Very few de novo mutations have been detected (Ashton-Prolla *et al.;* 2000) (Oliveira de Alencar *et al.;* 2014)



	Ex1	IVS1	Ex2	IVS2	Ex3	IVS3	Ex4	IVS4	Ex5	IVS5	Ex6	IVS6	Ex7
Missense	71		57		55		21		61		89		61
Nonsense	8		12		8		2		12		11		14
Indel	19		11		5		5		16		18		44
Splicing	1	1	0	3	2	4	1	7		5		4	
others									1				1

Figure 35. General distribution of *GLA* mutation correlated to Fabry's disease. Others: more mutations in the same patients

4.4.2 Patient 24

Case 24 was a 7 years old girl affected by Cantù's syndrome. Genetic testing was performed by next generation sequencing and the patient was found to carry the *ABCC9*c.C3460T,p.R1154W mutation. This mutation was already descripted in previous studies (Harakalova *et al.;* 2012) (van Bon *et al.;* 2012). A literature review about *ABCC9* mutations was carried out using public database such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and Pubmed (https:// www.ncbi.nlm.nih.gov/pubmed/). In total 28 ABCC9 pathogenetic mutations have been already reported (Fig.36). Among 16 different pathogenetic mutations, 13 are correlated to Cantu's syndrome (Grange *et al.;* 2006) (Scurr *et al.;* 2011) (Harakalova *et al.;* 2012), 2 refer to dilatative cardiomyopathy (Bienengraeber *et al.;* 2004) and 1 is correlated to the familial atrial fibrillation-12 (ATFB12, OMIM 614050) (Olson *et al.;* 2007). Furthermore, other 12 mutations in the *ABCC9* gene have been prevoiously reported as"probably pathogenetic" or "variants with uncertain significance". Notably, most of the *ABCC9* mutations resided within exon 27 of *ABCC9.*

ABCC9 mutations correlated to primary hypertrophic cardiomyopathy

ABCC9 mutations associated to Cantù's syndrome

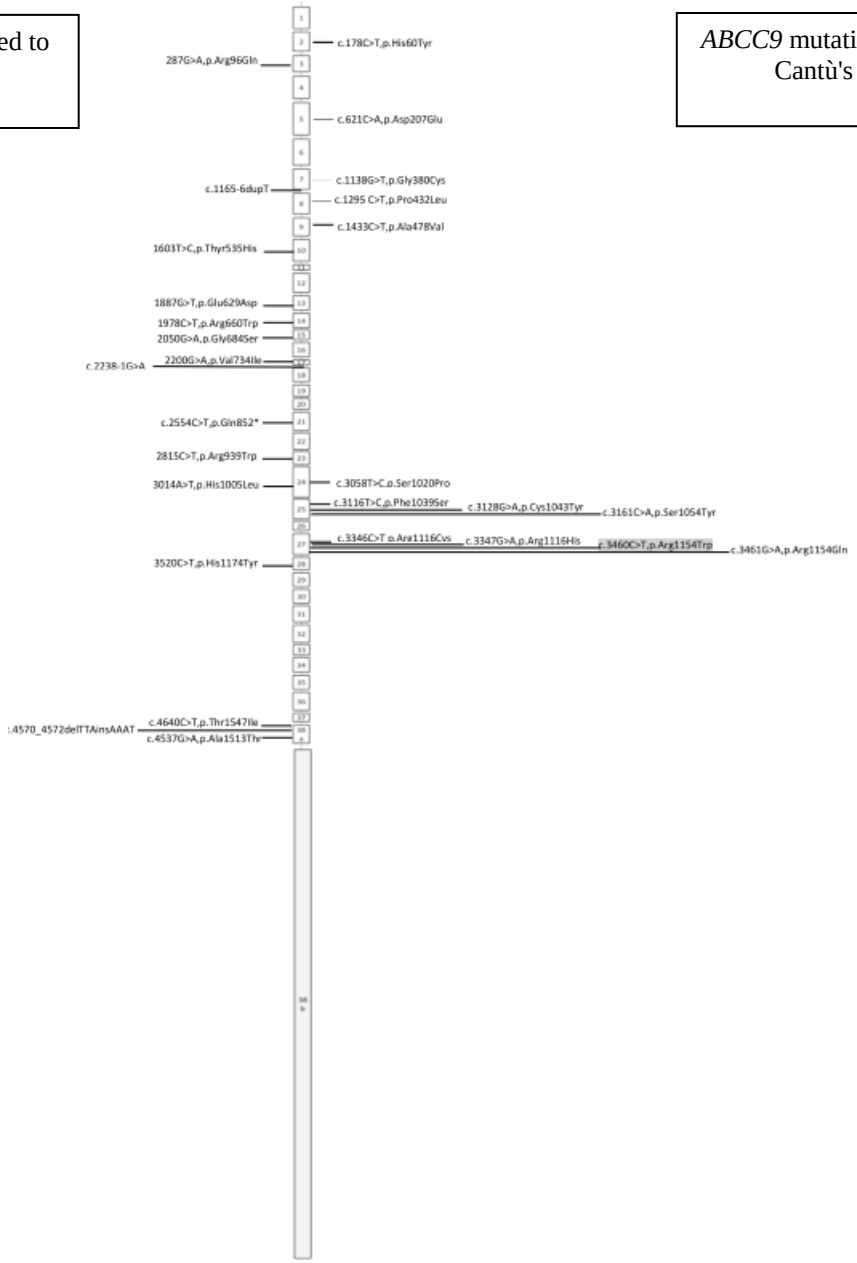


Figure 36. Schematic structure of *ABCC9* gene and known mutations associated to HCM and Cantù's syndrome White exons: NM_005691.3. Exons are in scale; introns are not in scale. Grey exons: NM_020297.3. To date, the discovered mutations map on the entire sequence. The mutation c.C3460T, p.R1154W mapped on exon 27 (in grey)

4.4.3 Patient 27

The patient, a 23-year-old female, was the only child born to unrelated Italian parents with a family history of cardiac disease (Fig.37). Her living father had developed chronic ischemic heart disease following a myocardial infarction at 52 years of age, while her mother began experiencing symptoms of progressive heart failure during the fourth decade of life, with the first hospital admission at age 39 with an ejection fraction (EF) lower than 30%. The diagnosis of idiopathic

dilated cardiomyopathy was made in this woman who died 5 years later due to a cerebral hemorrhage complicating the implant of a ventricular assist device. Since childhood, the patient had manifested mild intellectual impairment. At the age of 20, she was referred to the cardiomyopathy unit, division of cardiology and cardiac arrhythmias, of our institution for supraventricular tachyarrhythmias.

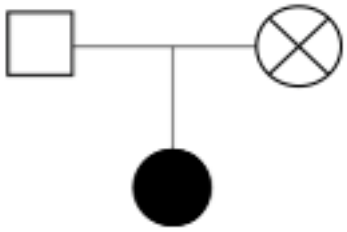


Figure 37. Familial pedigree of patient 27. The mother was affected by diatative cardiomyopathy. The father had a myocardial infarction at 52 years of age and the mother died at 54 years of age for a cerebral hemorrage complication

Electrocardiogram exhibited a normal sinus rhythm with a preexcitation pattern (Fig.38A). Echocardiography revealed severe cardiomyopathy with symmetric hypertrophy, small ventricular cavity, and mild EF reduction (55%) (Fig.38B). The ventricular septum thickness was quite high (32 mm) as well as the posterior wall (28 mm). She underwent cardiac radiofrequency ablation of the accessory pathway. During follow-up, she complained of dyspnea and general weakness. A cardiac magnetic resonance (CMR) confimed the hypertrophic phenotype, showing a decrease in the EF (45%). Holter ECG revealed nonsustained ventricular tachycardia, requiring an implantable cardioverter defibrillator. Despite medical therapy, left ventricle function progressively decreased. At the age of 22, echocardiography showed a hypokinetic and dilated LV with an EF of 30%. Notably, LV hypertrophy was reduced compared to previous evaluations (ventricular septal thickness of 18 mm).

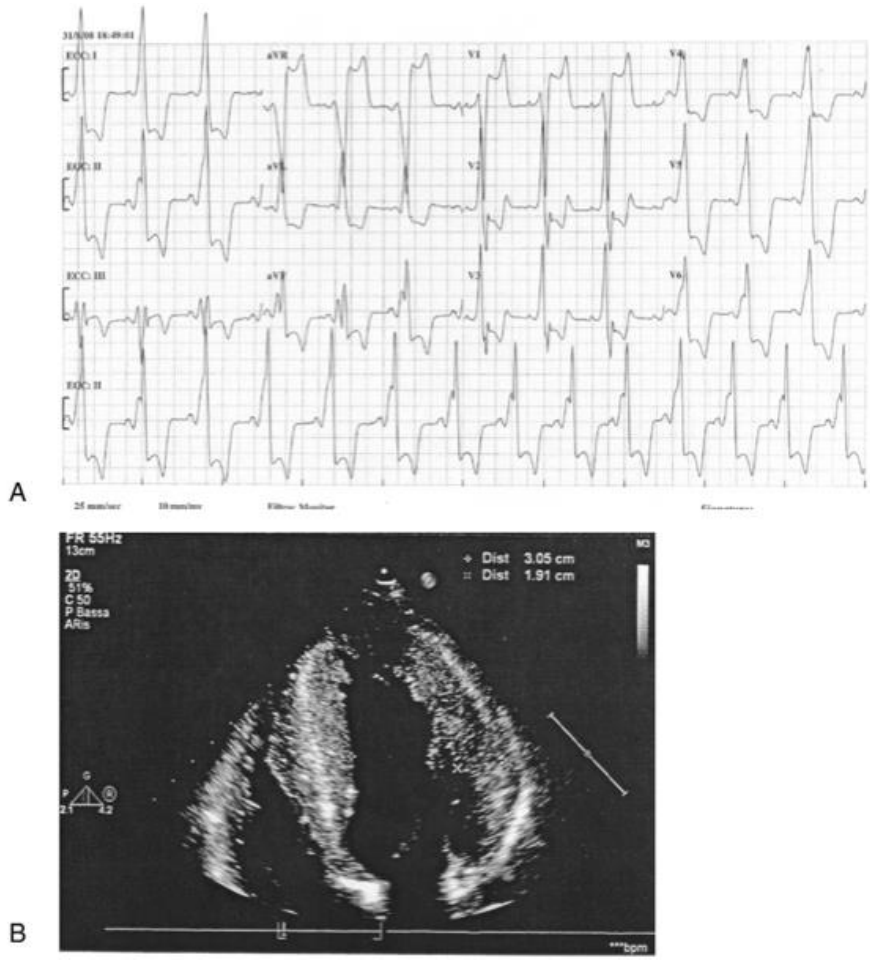


Figure 38. Echocardiographic and electrocardiographic features of the patient. (A) Echocardiogram showing marked left ventricular hypertrophy involving interventricular septum and lateral wall with increased echogenicity of the myocardium. (B) Rest 12-lead electrocardiography showing sinus rhythm with large delta waves due to overt ventricular preexcitation associated with markedly abnormal ventricular repolarization (Bottillo *et al.;* 2016c)

One year later, the patient complained of profound muscular weakness. Serum CK levels were within the normal range. An echocardiogram confirmed a low EF (30%). Due to the severe muscular weakness, a deltoid muscle biopsy was performed, which showed normal morphologic features of myocytes (Fig.39A-B). However, increased phosphatase reactivity was observed in some muscle fibers. This finding, in light of the association of cardiomyopathy, myopathy, and mild mental retardation, suggested the diagnosis of Danon disease (DD). Immunofluorescence for LAMP2 protein was negative in about 10% of fibers, as compared to normal control muscle (Fig.39C-D), prompting a genetic evaluation to confim the suspicion.

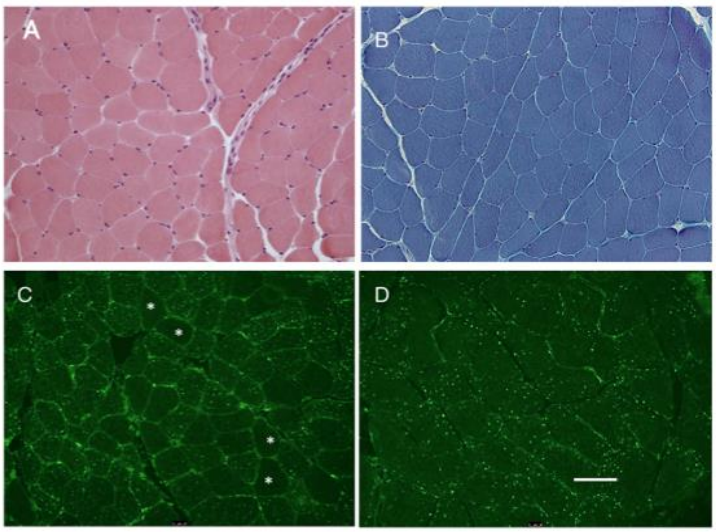


Figure 39. Histopathology of the left deltoid muscle. (A) Hematoxylin and eosin stain; (B) Gomori's trichome stain; (C) Immunohistochemistry of LAMP2 protein in the patient's muscle sample. LAMP2 negative fibers are marked with asterisk. (D) Immunohistochemistry of LAMP2 protein in an aged-matched control. Bar corresponds to 20 μm (Bottillo *et al.;* 2016c)

4.4.3.1 Molecular genetic analysis and analysis of genotype-phenotype correlations

The patient was found to carry two novel heterozygous nonsynonymous rare variants: a truncating mutation in *LAMP2* (chrX:g.119582928delA, NM_013995:c.453delT, p.F151Lfs*32) (Fig. 40A and B) and an intronic alteration in *LMNA* (chr1: g.156108272T>C, NM_001257374.1:c.1363-7T>C).

The identified *LAMP2* mutation allowed the definitive diagnosis of DD, while in silico studies did not disclose any functional role for the LMNA alteration. The *LAMP2* c.453delT alteration was not found in blood leukocytes from her father. Thus, based on the clinical history of her deceased mother, a maternal inheritance was suspected.

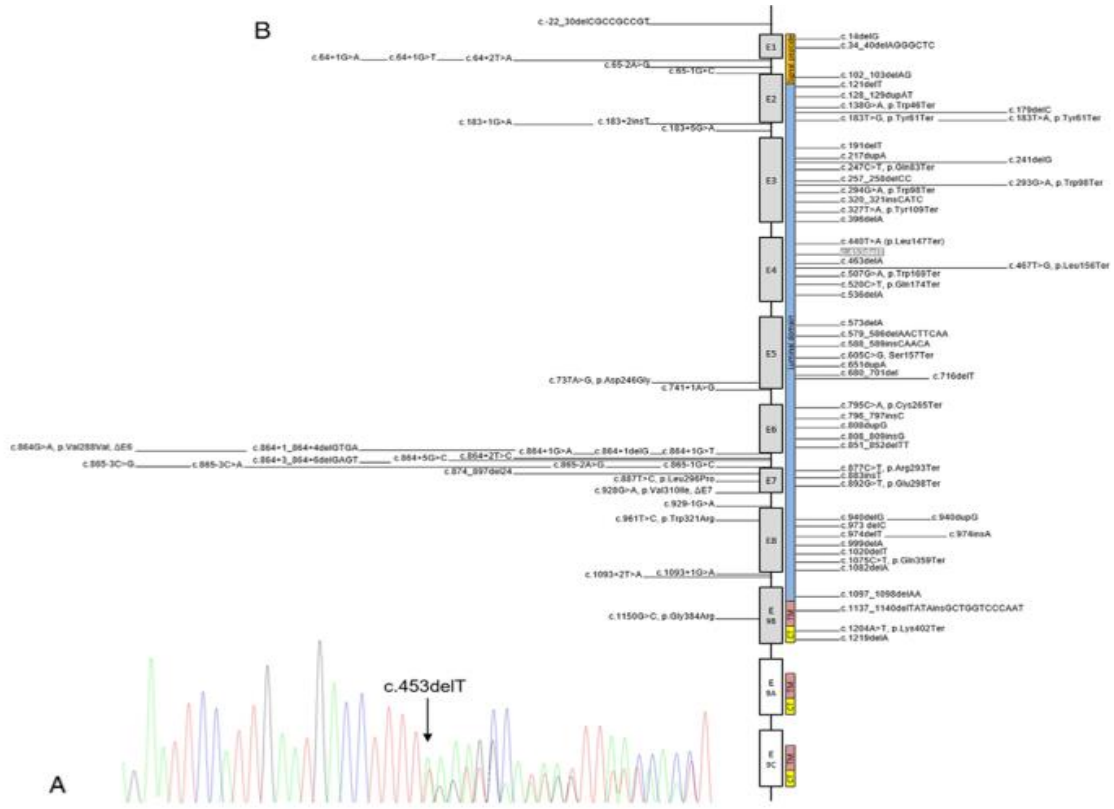


Fig. 40. Molecular studies of patient 27 (A) DNA sequence chromatogram of the LAMP2 c.453delT mutation found in the patient. (B) Schematic structure of *LAMP2* gene and known mutations associated to Danon disease. Exons are in scale; introns are not in scale. Exons belonging to isoform LAMP2B (NM_013995) are shown by grey rectangles. Exons 9A and 9C belonging, respectively, to isoforms LAMP2A (NM_002294.2) and LAMP2C (NM_001122606) are represented by white rectangles. The protein's functional domains are shown as colored boxes on the right of the exons. The nomenclature of each genomic change is given according to NM_013995, and the mutation found in the present patient is shown with a grey background. Truncating mutations (i.e., nonsense and frameshift) are shown on the right, while splicing, nonsynonymous, and synonymous alterations are shown on the left. The c.864GNA, p.Val288Val and the c.928GNA,p. Val310Ile were previously proven to cause the skipping of, respectively, exon 6 and exon 7. E: exon; TM: transmembrane region; CT: cytoplasmic tail (Bottillo *et al.;* 2016c)

Indeed, figure 40B shows exon/intron structure and the alignment of all the known point mutations mapping in *LAMP2*. In total, 68 *LAMP2* mutations have been reported in the literature (Danon *et al.;* 1981) (Nishino *et al.;* 2000) (Sugie *et al.;* 2002) (Balmer *et al.;* 2005) (Fanin *et al.;* 2006) (Spinazzi *et al.;* 2008) (Kim *et al.;* 2010) (Boucek *et al.;* 2011) (Dara *et al.;* 2011) (Cheng *et al.;* 2012) (Eistein *et al.;* 2012) (Hedberg *et al.;* 2015) (Bottillo *et al.;* 2016a) (Bottillo *et al.;* 2016b) (Bottillo *et al.;* 2016c) and 16 additional sequence alterations have been annotated as "pathogenic" or "likely pathogenic" in the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/). The mutations distribute along the entire gene sequence without evidence of hotspot nucleotides. The majority of sequence alterations map from exon 1 to exon 8, affecting all three LAMP2 isoforms. However, most of the splice site alterations map in intron 6 (Nishino *et al.;* 2000) (Bui *et al.;* 2008) (Cottinet *et al.;* 2011) (Hedberg *et al.;* 2015), and none of theme maps in introns 3 or 4.

Based on a review of published genetic and clinical data, we statistically tested some genotype–phenotype correlations using chi-square test and two-tailed Student's t test to compare, respectively, categorical and continuous variables. The type and position of the mutations were used to categorize the genotype, while the following categories were used as a measure of phenotype severity: age of symptoms' onset, age of death, levels of serum CK, heart transplantation, WPW, cardiomyopathy, skeletal muscular weakness, pacemaker (PMK), ICD, and intellectual disability. We found a statistically significant correlation between mutations located in LAMP2 exons 1 to 8 and a severe phenotype. Patients harboring alterations mapping in exons 1 to 8 showed indeed a higher frequency of WPW syndrome (P=0.04) and cardiomyopathy (P=0.0001), compared to patients mutated in exon 9B. Mutations in exons 1 to 8 actually alter all the three LAMP2 isoforms, while alterations of exon 9B are restricted to LAMP2B transcript. We then investigated the correlations between the type of mutation (i.e., truncating, missense, or splicing) and the clinical outcome. The implantation of PMK was correlated with the type of mutation, with PMK implanted in 6%, 12%, and 26% of patients with truncating, missense, or splicing mutations, respectively (P=0.05). In addition, we found that missense alterations correlated with a lower incidence of cardiomyopathy (Pb.0001). Finally, as previously described, we confirmed that truncating mutations show the earliest age of symptoms' onset (18±11 years). DD cases carrying splicing mutations tended to have onset later in life (20±13 years), while missense mutations showed the latest age of onset compared with all other mutations (32±23 years) (P = 0.04).

4.4.3.2 Morphological and immunohistochemical analysis of explanted heart

One year later, at the age of 23, the patient experienced severe heart failure and underwent orthotopic cardiac transplantation. Immediately after explant, the heart was weighed and photographed. Myocardial samples were obtained from multiple sites in both ventricles and were either processed for routine histology or snap frozen in liquid nitrogen-chilled isopentane for histochemical, biochemical, and molecular studies (in collaboration with Prof.ssa d'Amati, Dept. of Radiology, Oncology and Pathology, Section of Pathology, Policlinico Umberto I, Sapienza University of Rome, Rome, Italy). On gross examination, the explanted heart showed severe asymmetric left ventricular hypertrophy, mainly involving the septum (septal/free wall thickness ratio of 1.7). Multiple small foci of myocardial scarring were present, extensively involving the subepicardial and midventricular layers of the anterior, lateral, and posterior aspects of the LV free wall. Conversely, the ventricular septum involvement was limited to the anterior portion and consisted of sparse foci of scarring, within the subendocardium (Fig.41A).

Cardiac valves and coronary tree were unremarkable.

At histology, cardiomyocytes were hypertrophic and showed sarcoplasmic vacuoles filled with Periodic Acid–Schiff (PAS)-positive granules (Fig.41B-C). Multiple foci of myofiber disarray with increased interstitial collagen were present both in the anterior wall and the septum (Fig.40D). The areas of replacement fibrosis in the LV free wall and septum showed a close spatial relationship with remodeling of intramural coronary arterioles, consisting of medial wall thickening due to smooth muscle cell hypertrophy and increased collagen deposition, with variable degrees of intimal thickening and perivascular fibrosis (Fig.41E-F) leading to severe lumen narrowing.

Analysis of LAMP2 expression by immunohistochemistry, performed both on the LV and Right Ventricle (RV) free walls and on the septum, revealed a mosaic pattern consisting of discrete clusters of either stained or unstained cardiac myocytes (Fig.42A) Cells lacking LAMP2 were particularly frequent in the septum, accounting for about 60% of cardiac myocytes. At electron microscopy (EM) analysis cardiomyocytes showed disorganized myofibrils and autophagic vacuoles containing myeloid bodies, electrondense granular material, and cytoplasmic debris. These vacuoles were surrounded by a single membrane. In addition, accumulation of free and vacuole bound glycogen was observed (Fig.42C-F).

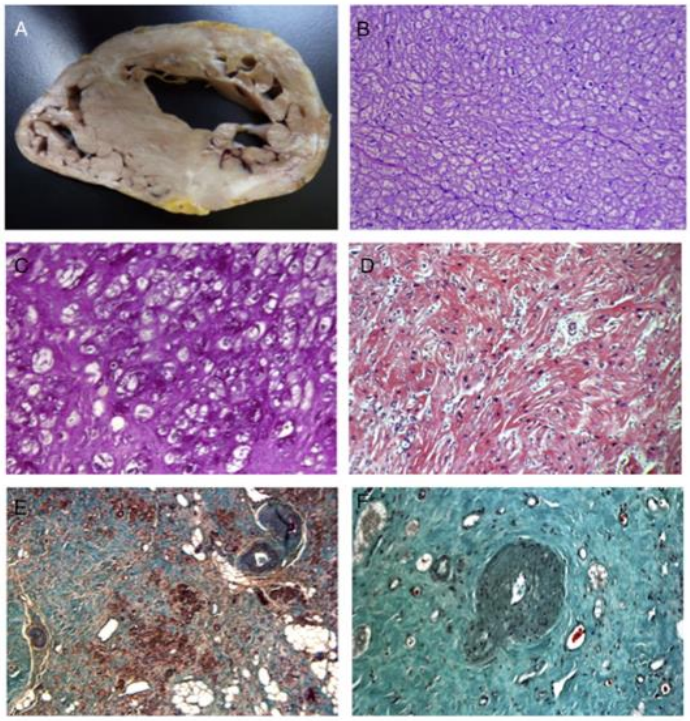


Figure 41. Macroscopic and histologic features of explanted heart. (A) Short-axis section of the heart showing biventricular, asymmetrical hypertrophy, mainly involving the septum (septum thickness = 23 mm; free wall LV thickness = 13 mm), with mild chamber dilation. LV free wall shows multiple foci of subepicardial and midventricular scarring. (B) Cardiac myocytes with prominent sarcoplasmic vacuoles (hematoxylin and eosin, original magnification x4). (C) The vacuoles contain PAS-positive granules (PAS, original magnification x20). (D) Myocardial disarray in the septum (hematoxylin and eosin, original magnification x10). (E) Remodeling and lumen narrowing of intramyocardial

arterioles associated with foci of scarring (Masson trichrome stain, original magnification x10). (F) Higher magnification of a remodeled vessel showing medial wall smooth muscle hyperplasia. (Masson trichrome stain, original magnification x40) (Bottillo *et al.;* 2016c)

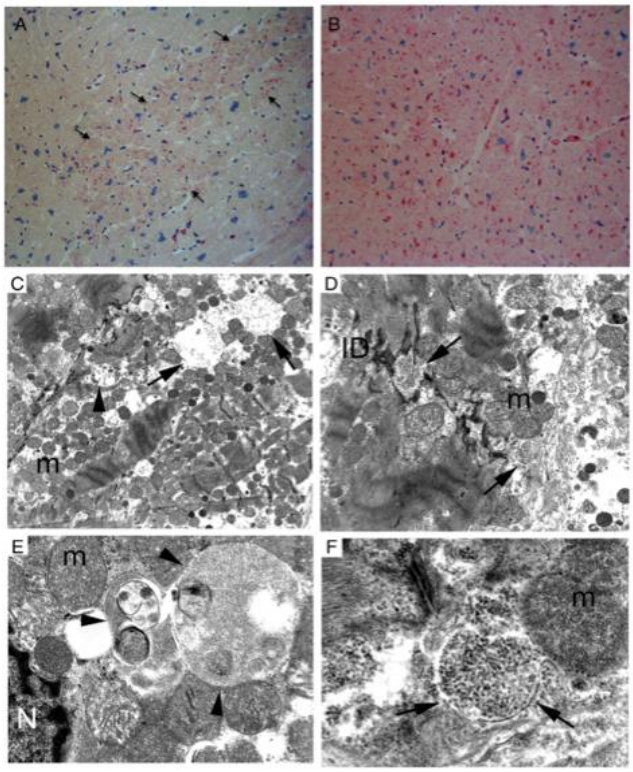


Figure 42. LAMP2 protein expression in cardiac myocytes. (A) Immunohistochemistry on the patient's heart shows discrete clusters of either stained (red) or unstained cardiac myocytes. Note a cluster of stained cardiac myocytes bordered by arrows. (B) Normal pattern of LAMP2 staining in a control heart (original magnification x20). (C-F) EM analysis of myocardium. (C-D) The photographs show myofibrils and intercalated discs disorganization. Autophagic vacuoles (arrowheads) and glycogen (arrows) are accumulated in cardiac myocytes. ID: intercalated disc, m: mitochondria. Original magnification: x5900 and x8900, respectively. (E) Higher magnification of autophagic vacuoles in a cardiac myocyte (arrowheads). Original magnification: x21,000. (F) Higher magnification of vacuoles containing glycogen (arrows). Original magnification x29,000 (Bottillo *et al.;* 2016c)

4.4.3.3 Analysis of the X chromosomes methylation status

Considering the severity of the disease manifestations and progression, an assay for evaluating the methylation status (i.e., inactivation) of the X chromosomes (XCI) was performed on DNA both from blood leukocytes and cardiac muscle obtained from the LV, RV, and the septum of the patient's explanted heart. The same test was also performed on her father's DNA in order to establish the parental inheritance of each X chromosome. The XCI pattern was determined by evaluating the cytosine methylation of CpG dinucleotides within the polymorphic Cytosine, Adenine and Guanine (CAG) repeat in the first exon of the androgen receptor (AR) gene, located on chromosome X (Warburton *et al.;* 2005) (Elstein *et al.;* 2012) (Bottillo *et al.;* 2016c).

The inactivation rate of the X chromosome maternally inherited (assumed to carry the c.453delT LAMP2 mutation) was 62% in blood leukocytes of our patient. The random X-chromosome inactivation found in the cardiac sections and leukocytes of patient 27 excluded the possibility that selective involvement of DNA both from blood leukocytes and cardiac muscle obtained from the LV, RV and the septum is due to skewed X-chromosome inactivation.

5. DISCUSSION

This study reports a next generation analysis of the prevalence of sarcomeric and non-sarcomeric gene variants in 41 patients affected by primary hypertrophic cardiomyopathy, and in 3 cases affected by syndromic HCM. This work was aimed the discovery of disease causing genetic variation for an appropriate genetic counseling, as well as for the enlargement of the hypertrophic cardiomyopathy mutational spectrum thorough a broad genetic test.

The employed high throughput sequencing technology enabled the generation of large amounts of sequence data, and determined the need of an accurate assessment of the identified genetic variations. The experimental characterization of all the observed DNA changes would have support the functional and/or regulatory impact of the various mutations, but it requires a long experimental time that was impractical in our clinical setting. Likewise, co-segregation analysis of the DNA changes within families would have been appropriate, but it is uninformative in small pedigrees and it is hindered by HCM incomplete penetrance and variable expressivity.

A high number of bioinformatics solutions for the annotation, scoring and classification of variants are currently available to address the challenge of predicting the functional consequences of a mutation. Our results show the relevance of incorporating integrated computational workflows to predict the biomedical impact of the various DNA variants resulting from a NGS approach, and to identify functionally significant or clinically actionable variants.

After the initial quality assessment of the sequencing reads, their alignment with the reference genome, and the subsequent variant calling, the resulting DNA changes were annotated for facilitating the filtering and prioritization steps. Some online free sequence databases such as 1000 Genome Project, NHLBI-ESP 6500 exome projects and dbSNP138, were helpful in the annotation process. Since all the patients included in this study were Italian, we checked the frequency of each variant both in 1000 Genomes Project Global and Exome Sequencing Project Global, but also in 1000 Genomes Project European. Based on those datasets we considered only variants with an allele frequency ≤ 0.01. Sanger sequencing was employed to rule out false positive calls from all the filtered and prioritized DNA changes. These analyses generated a subset of variants presenting several data interpretation challenges, but also some interesting genotype–phenotype cues. For further delineating the likelihood to be disease-relevant nsSNVs, we hence established a bioinformatics framework for the assessment of each variant functional role, based on previously reported data and in silico predictions. We evaluated the performance of several different independently published methods that aim to predict the functional consequences of alleles that result in amino acid substitutions. Moreover, the impact of a sequence variant with respect to the

evolutionary conservation was derived from genomic evolutionary rate profiling (GERP) score, phylogenetic P-value (PhyloP) score and PhastCons score. Those predictions aimed to hypothesize the probable impact of a particular genetic variant on function or regulation and suggested that most of the identified nsSNVs have the potential of being functionally pathogenetic.

Several free online tools (i.e. SIFT, Polyphen 2_HDIV, Polyphen2_HVAR, Provean, LRT, Mutation Taster, Mutation Assessor, FATHMM, RadialSVM, LR, CADD v1.3) were employed to assess sequence- and structure-based features (Bottillo *et al.;* 2016b). Provean gave back a prediction score for every input variant. Between the other tools, LRT failed more frequently to give a result (16 not predicted variants out of 85 different ones). Regarding our set of DNA changes, Mutation Taster and Mutation Assessor were the methods resulting respectively in the greater and in the fewer number of deleterious predictions. We therefore observed that combining multiple prediction tools provides a more even balance between sensitivity and specificity than most of the individual methods.

Besides the score-based predictions, performing molecular modeling gave us the opportunity of visually and directly testing tertiary or quaternary structure. The location of a coding SNV with respect to the surface-interior or interface of the protein structure-could indeed influence disease manifestation.

Our NGS approach of 62 sarcomeric and non-sarcomeric genes identified likely pathogenic sarcomeric variants in 58% non-syndromic patients, consistent with previous studies that have used both conventional genetic sequencing techniques (Marian *et al.;* 2001; (Arad *et al.;* 2002b); (Hershberger *et al.;* 2013), and a NGS approach in larger cohorts. In particular, in 2013 Lopes and coauthors analyzed 223 unrelated cases for 41 sarcomeric and non-sarcomeric cardiovascular genes and found that 121 patients (54%) carried alterations in 9 sarcomeric genes (including *ACTC1*, *MYPBC3*, *MYH6*, *MYH7*, *MYL2*, *MYL3*, *TNNI3*, *TNNT2* and *TPM1*) (Lopes *et al.;* 2013). In our study we found alterations in two additional sarcomeric loci (i.e. *MYOM1* and *NEBL*) not analyzed by Lopes and colleagues, maybe explaining our slight increased rate of cases with sarcomere gene mutations.

Inclusion of not only sarcomeric loci, but also of many different genes implicated in cardiomyopathies, resulted in the identification of several rare (frequency ≤ 0.01) DNA changes. We found that 82% of the patients harbored at least one rare nsSNV. Our results confirm the well-established role of *MYBPC3* a major gene in the HCM pathogenesis (Millat *et al.;* 2010); (Lopes *et al.;* 2013), but also show that the HCM mutational spectrum consists mainly in missense mutations. In fact, only ~7% of the identified variants are truncating (frameshift or nonsense SNVs), and they map preferentially in *MYBPC3* (Bottillo *et al.;* 2016a). This finding suggests that *MYBPC3*

molecular alterations can be both amino acid substitutions but also loss of function mutations, in contrast to the other analyzed loci that resulted mostly affected by missense change. We did not observe any strong mutation hot-spot, and only 8/106 nsSNVs recurred in more than one patient.

In our cohort, sarcomeric loci resulted as the most affected ones and, among them *MYBPC3*, *MYH6* and *MYH7* showed the main proportion of nsSNVs. These data confirm the major role of cardiac myosin binding protein C and of myosin heavy chain 7 in hypertrophic cardiomyopathy pathogenesis (Millat *et al.;* 2010; Lopes *et al.;* 2013), and following the results by Lopes et al. (2013) clench myosin heavy chain 6 as one of the most recently established sarcomeric HCM genes. Eleven percent of the patients showed only sarcomere nsSNVs, while 20% of cases harbored at least one sarcomeric nsSNV with at least a desmosomal one. Moreover we found that 14% of the HCM patients displayed at least one desmosomal nsSNV but no other sarcomere change. Such a direct association of desmosomal alterations with HCM has not been described to date (Bottillo *et al.;* 2016a). Four/40 patients resulted negative to the next generation analysis about 62 sarcomeric and non-sarcomeric genes. To date, there are not any strong algorithms enable to detect large duplications/deletions using NGS data. Genomic alterations in genes commonly associated with hypertrophic cardiomyopathy are an infrequent but important genetic cause of HCM in ~2% of patients without identified mutations by sequence analysis.

Therefore, we hypothesized that large deletions or duplications of one or more selected genes could occur in our 4-HCM patients in which no mutation was identified by NGS analysis.

In 2013, Lopes and colleagues analyzed by NGS a large cohort of HCM cases and found that 10% of them carried nsSNVs in titin only in association with desmosomal or ion channel variants, but not other sarcomere ones. Titin variants have previously been reported in apparently healthy patients but we found that 52% of the 44 HCM patients harbored at least one *TTN* nsSNVs with MAF ≤ 0.05. Indeed, high prevalence of titin mutations was also reported by Golbus and colleagues who analyzed the '1000 Genomes' cohort. A cumulative frequency of titin indels of 9% was found, with just over 5% of the general population having a 18bp in frame deletion in the PEVK region of titin (Yamasaki *et al.;* 2001) (Castro-Ferreira *et al.;* 2010). It is not to be underestimated the regulation of titin by calcium ions. The calcium ion (Ca^{2+}) has a big role as regulator of cardiomyocyte distensibility, especially due to the modulation of the interaction between titin and the thin filaments. Studies have shown that the PEVK domain in the extensible region of the N2B isoform binds to F-actin and that this interaction can contribute to the passive rigidity of cardiomyocytes (Yamasaki *et al.;* 2001) (Castro-Ferreira *et al.;* 2010). Although Ca^{2+} alone can not interfere with this binding, when this ion binds to protein S100A1 (S100 calcium-binding protein A1), which is found at high concentrations in the myocardium, this interaction is inhibited,

decreasing the rigidity of the cardiomyocyte. The observation of the increase in rigidity of the cardiac muscle in parallel to the decrease in the concentration of Ca^{2+} during diastole supports this mechanism (Yamasaki *et al.;* 2001) (Castro-Ferreira *et al.;* 2010). Thus, titin may be an important disease gene not only because it causes diseases on its own but it may modify the phenotype of mutations in other genes.

Future study on the *TTN* changes discovered in our 44 HCM patients, with the employment of programs for assessing the possible pathogenicity of these variants, might prove the role of this giant protein as a phenotypic modifier in association with rare mutations discovered by the NGS panel in the pathogenesis of hypertrophic cardiomyopathy.

Furthermore, mutations in the filamin C gene (*FLNC*) have very recently been associated with HCM (Valdes-Mas *et al.;* 2014). Moreover, some of those cases might also have alterations of genomic region that were not covered by our NGS panel (i.e. 3'UTR, 5'UTR, ncRNAs). Future studies of *FLNC* and of sequences regulating the cardiomyopathies genes' expression will shed some light on rising role of desmosomes in HCM as well as in DCM (Haas *et al.;* 2015).

As sarcomere and desmosomes nsSNVs were the major changes associated with HCM, and we speculate that the other non-sarcomeric loci might have a modifying effect on HCM phenotype. As demonstrated in a recent study, non-sarcomeric variants may indeed influence the disease expression, outlining the complexity of HCM genetic basis (Lopes *et al.;* 2014). Statistical analyses performed in our cohort, revealed that not only a higher number of nsSNVs seems to correlate with an earlier disease onset, but also that those alterations in genes for Ca^{2+}homeostasis, for K+ and Na+ channels, and for cytoskeletal proteins can modulate HCM expression. Ion (Ca^{2+}, Na+ and K+) channel variants correlate to an earlier disease onset and to a lower maximal wall thickness, while changes in cytoskeletal loci correlate with non-sustained ventricular tachycardia onset and low-grade diastolic dysfunction. Moreover, sarcomeric nsSNVs were correlated to a lower NYHA class (Bottillo *et al.;* 2016a). A recent study on a large HCM population, reported that the prevalence of male sex was lower in sarcomere-positive individuals (Lopes *et al.;* 2014). We did not replicate this finding, and we found a higher proportion of males respect to women in the group of positive nsSNVs cases. The comparison of data from familial and sporadic cases generated 3 significant correlations: (i) the mean of the maximum wall thickness value of was lower in the sporadic group; (ii) non-sustained ventricular tachycardia was correlated with familial cases; (iii) the implantation of ICD resulted to be linked to a familial framework.

We also identified 49 synonymous rare variants that might act as phenotype modifiers. Additional studies about their function on mRNA transcription, splicing, transport, translation or modification are required for determining their possible non-silent role.

Among the 97 identified nsSNVs, a single variant was identified in homozygosity (i.e. *ABCC9* p.V734I in patient 21). Such a variant was already associated to a higher risk of developing precocious myocardial infarction (Minoretti *et al.;* 2006), but patient 21 did not show that occurrence up to now.

Including both the non-syndromic and the syndromic cases, 25 out of 62 genes resulted negative to the mutation screening. Between them, there were not desmosomal loci, but four sarcomeric ones: *ACTC1*, *MYL3*, *TNNC1* and *TNNI3*. In line with the present results, previous studies reported a rate of heterozygous mutated patients less than 1% for *ACTC1* (Olson *et al.;* 2000), *MYL3* (Poetter *et al.;* 1996) and *TNNC1* (Landstrom *et al.;* 2008). *TNNI3* alterations are expected in about 5% of HCM cases (Hershberger *et al.;* 2013), but we did not found any mutated patient for this gene. If a clear *TNNI3* genotype-phenotype association exists, this discordance might be due to a bias in patients' selection, but of course additional studies on larger cohorts are needed to investigate this observation.

Between the 25 negative genes, there were 16 loci already associated with hypertrophic or dilated cardiomyopathy (*ACTC1*, *ANKRD1*, *CRYAB*, *DES*, *EYA4*, *GATAD1*, *LDB3*, *MYL3*, *MYLK2*, *MYOZ2*, *NEXN*, *PLN*, *SGCD*, *TCAP*, *TNNC1* and *TNNI3*), 3 with myopathies and neuromuscular disorders (*EMD*, *FXN* and *TAZ*), 2 with storage disorders (*PRKAG2* and *TTR*), one with ventricular tachycardia (*CASQ2*), one with arrhythmogenic right ventricular cardiomyopathy (*TMEM43*), one with Noonan syndrome (*PTPN11*) and one cardiovascular candidate gene (*CTF1*). Six of the negative loci (*ANKRD1*, *CRYAB*, *CTF1*, *EMD*, *EYA4* and *FXN*) were not included in a recent study about NGS analysis of a large cohort of HCM patients (Lopes *et al.;* 2014).

Three patients showed syndromic HCM and the NGS analysis allowed the confirmation of their clinical diagnosis. Patient 10 was a 60-year-old woman with apparently isolated non-obstructive HCM featuring a maximum left ventricular wall thickness of 18 mm. Subsequently, she resulted heterozygous carrier of the p.N215S mutation in *GLA* gene, responsible of the X-linked recessive Fabry disease. This mutation was previously associated to a cardiac variant of the disease by Eng et al. (1993) and by Davies et al. (1993), and our data confirm this genotype–phenotype association. Patient 24 was a 6-year-old girl originally ascertained for the clinical suspect of Cantù syndrome, a syndromic form of early-onset HCM also featuring coarse face, hirsutism, persistence of fetal circulation, overgrowth of prenatal onset and mild bone dysplasia, which was recently associated with specific *ABCC9* mutations (van Bon *et al.;* 2012). Accordingly, we found the recurrent heterozygous *ABCC9* p.R1154W mutation, which subsequently resulted de novo (Harakalova et al., 2012; van Bon et al., 2012). Finally, patient 27 showing some intellectual impairment and severe cardiac disease requiring heart transplant and limb weakness, harbored the novel p.F151Lfs*32

mutation in *LAMP2*, the gene causing Danon's disease, an X-linked dominant disorder predominantly affecting cardiac muscle (Nishino *et al.;* 2000). According to our meta-analysis of published clinical data (summarized in Table 12), the majority (81%) of cases are familial. To date, characterization at both the clinical and molecular levels has been reported for 124 Danon patients (60 males, 31 females, and 33 cases for which sex has not been reported).

	Men (n=60)	Women (n=31)	Present patient
Age at symptoms' onset	16 years (±11)	27 years (±15)	15 y
Cardiomyopathy	45	27	+
WPW	23	5	+
ICD	16	7	+
Age at ICD implantation	21 y (±9)	39 y (±15)	21 y
PMK	8	3	−
Age at PMK implantation	28 y (±14)	44 y (±14)	−
Learning and cognitive disabilities	26	1	+
Ocular	11	3	−
Skeletal muscular weakness	39	4	+
Elevated serum CK levels	49	3	−
Heart transplantation	11	6	+
Age at heart transplantation	24 y (±8)	32 y (±16)	23 y
Death	13	10	−
Age at death	20 y (±5)	45 y (±6)	−

y: years.

Table 12. Clinical features of patient 27 and other 91 additional cases from previous studies (Bottillo *et al.;* 2016c)

Heart disease is the dominant clinical feature in both males and females affected by Danon disease, and it is often associated with conduction disease. Early-onset, severe cases have only occasionally been described in females; the onset of cardiac symptoms is usually 10 years later in affected women as compared to males. This is typically ascribed to most women expressing a functional copy of *LAMP2* (haplosufficiency) that is sufficient to protect them even with X-inactivation. Accordingly, life expectancy is about 25 years longer, and heart transplantation is performed about 8 years later in females than in males. The early onset of cardiac symptoms in the present case could be accounted by the presence of a truncating mutation, as shown in our analysis of published literature. However, only a few female patients carrying LAMP2 mutations with both early and severe cardiac disease have been reported to date (Yang *et al.;* 2005) (Dara *et al.;* 2011) (Miani *et al.;* 2012) (Hedberg *et al.;* 2015) (Bottillo *et al.;* 2016c), and the reasons for this unusual phenotypic expression are not completely understood. Oldfords and colleagues (Hedberg *et al.;* 2015) evaluated the distribution of LAMP2-deficient cardiac myocytes in hearts from three female Danon patients, which were transplanted due to severe cardiomyopathy. They hypothesized that the inhomogeneous distribution pattern, rather than the absolute amount of LAMP2-deficient cardiac myocytes, is responsible for the severe cardiac involvement in these cases. In our study, we extend their results by showing that the uneven, patchy distribution of LAMP2 in cardiomyocytes is correlated with the percentage of XCI, being more extensive in the ventricular septum. Moreover, we demonstrate a previously unreported finding in Danon cardiomyopathy, that is, the presence of

diffuse and severe lumen narrowing of coronary intramural arterioles, with a clear spatial relationship with foci of replacement-type fibrosis. This association has been extensively described in hypertrophic cardiomyopathy due to mutations in sarcomeric protein genes and is considered the morphologic substrate of progressive systolic impairment in hypertrophic cardiomyopathy patients due to ischemic myocyte loss (Olivotto *et al.;* 2015). The presence of diffuse microvascular disease could be a determinant for the severe cardiac phenotype in this female patient with DD. Intriguingly, the ventricular septum was relatively spared from myocardial scarring, while the left ventricular-free wall was diffusely affected, resulting in a marked decrease in the wall thickness, which mimicked the pattern of asymmetrical septal hypertrophy frequently described in sarcomeric HCM (Bottillo *et al.;* 2016c). Indeed, this case shows overlapping histologic findings with sarcomeric HCM, that is, extensive myofiber disarray and microvascular remodeling associated with microscarring raising the issues of the differential diagnosis between the two diseases also on pathologic examination.

Finally, the pattern of distribution of myocardial scarring in our case is in line with cardiac magnetic resonance findings of late gadolinium enhancement involving the LV free wall and sparing the septum, reported in a Danon patient (Piotrowska-Kownacki *et al.;* 2009), and may be helpful in the differential diagnosis between primary hypertrophic cardiomyopathy and Danon disease.

6. CONCLUSION

Cardiomyopathy refers to an important and heterogeneous group of related diseases of the heart muscle in which the myocardium is structurally and functionally abnormal such as hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic right or left ventricular cardiomyopathy (ARVC/ALVC) and restrictive cardiomyopathy (RCM). The evidence of genetic and phenotypic overlap among different cardiomyopathies adds other complexity, often resulting in stepwise analysis of multiple disease-specific genes when the diagnosis is not so clear.

Hypertrophic cardiomyopathy is the most common genetic cardiovascular disorder, affecting one of every 500 adultsaffects people of both genders and of various racial and ethnic origins. It is a primary genetic cardiomyopathy characterized by left ventricular hypertrophy, which is usually asymmetric, with greater involvement of the interventricular septum than the left ventricular-free wall in the abscence of another systemic or cardiac process.

Thanks to the employment of next generation sequencing technology in view of the genetic overlap between different types of cardiomyopathies, we developed a molecular test suitable for a broad series of both non-syndromic and syndromic affected patients. Moreover, despite the large amount of data coming out from an NGS protocol, we delineated a prompt informatic pipeline for the prioritization of the most likely pathogenetic variants in a clinical context.

The conducted genotype–phenotype correlations represent a starting point for expanding the present results to larger cohorts and for delineating the contribution of each analyzed gene in the onset and clinical variability of HCM. In the future indeed, a broad range of molecular causes (i.e. desmosomal or other non-sarcomeric alterations) and environmental factors will need to be investigated in wider populations of sporadic and familial cases.

Futhermore we analyzed the same 44 HCM patients for another cutom cardio panel designed to analyze coding, intronic junctions and UTR sequences of *TTN* genes. The discovered titin variants might not cause diseases on their own but may modify the phenotype of mutations in other genes. These data argue that *TTN* mutations should not currently be interpreted as disease causing in most situations, urge caution in the interpretation of these variants in clinical practice, and support the need for the development of functional assays that better assess pathogenicity.

If correct this would be an important consideration for future genetic testing and study of genotype-phenotype relationships.

Therefore we describe the clinical, pathological, and molecular features of a novel *LAMP2* c.453delT mutation in one of our HCM-related disorders patients affected by Danon disease. Our findings suggest that several features may contribute to the early and severe cardiac phenotype in

female DD patients. The type of mutation may account for the early disease onset, while both the inhomogeneous distribution of LAMP2 loss due to zonal inactivation of the X chromosomes and the presence of microvascular remodeling may be determinants in the rapid progression to heart failure.

In the context of cardiomyopathies, the characterization of the family mutation helps indeed in planning surveillance and early detecting possible complications in close relatives. This also implies that relatives discovered without the familial mutation can avoid unnecessary follow-up.

In conclusion, our results enlarge the mutational spectrum of hypertrophic cardiomyopathy patients with the intent of contributing to the definition of a molecular paradigm for explaining the diversity clinical spectrum of hypertrophic cardiomyopathy.

LIST OF ABBREVIATIONS

AC	Anelylylcyclase
AICD	Automated Implantable Cardioverter-Defibrillator
AF	Atrial Fibrillation
AR	Androgen Receptor
ARVC	Arrhythmogenic Right Ventricular Cardiomyopathy
ARVD	Arrhythmogenic Right Ventricular Dysplasia
ALVC	Arrhythmogenic Left Ventricular Cardiomyopathy
AMI/NSTEMI	Acute Myocardial Infarction Non-ST elevation Myocardial Infarction
AMP	Activated protein kinase
ANGII	Angiotensin II
ASD	Atrial Septal Defect
ATFB	Atrial Fibrillation
BMD	Becker Muscular Dysptrophy
BP	Blood pressure
BRGDA1	Brugada syndrome 1
BTHS	Barth syndrome
CAB	Coronary Bypass
CaChBl	Calcium channel blocker
CFCS	Cardiofaciocutaneous syndrome
CK	Serum creatine kinase
CMD1M	Cardiomyopathy, dilated, 1M
CMRI	Cardiac magnetic resonance
CMT2B1	Charcot-Marie-Tooth disease, axonal, type 2b1
Cond Abn	Conduction abnormalities
CPVT1	Ventricular Tachicardia, Catecholaminergic Polymorphic, 1
CPVT2	Ventricular Tachicardia, Catecholaminergic Polymorphic, 2
CS	Costello syndrome
CT	Cytoplasmic tail
CTRCT16	Cataract 16
CTS1	Carpal Tunnel syndrome
CV	Cardiovascular
CVG	Coronary-Ventriculography
DAD	Deafness, autosomal dominant
DCM	Dilated cardiomyopathy
DCWHK	Cardiomyopathy, dilated with woolly hair and keratoderma
Dgn Age	Age at diagnosis
Diast Dys	Diastolic Dysfunction
DM1	Myotonic dystrophy type 1
DMD	Duchenne Muscular Dystrophy
ECC	Excitation-Contraction Coupling
ECG	Electrocardiogram
ECG Hyp	Electrocardiogram Hypertrophy criteria
EDMD	Emery-Dreifuss Muscular Dystrophy

EF	Ejection fraction
ERT	Enzyme replacement therapy
ET	Endothelin
DD	Danon Disease
emPCR	Emulsion PCR
Fam His	Family History
FAP	Amyloidosis, hereditary transthyretin-related
FCT	Fetal cardiac titin
Fn3	Fibronectin type 3
FPLD2	Lypodystrophy, familial partial, 2
FRDA	Friedreich's ataxia
GC	Guanylylcyclase
HCM	Hypertrophic Cardiomyopathy
HD	Huntington's disease
HEM	Hemizygosity
HF	Heart Failure
HGPS	Hutchinson-Gilford Progeria
HMZ	Homozygosity
HOCM	Obstructive Cardiomyopathy
Hpt	Hypertension
HR	Heart Rate
HTX	Heart Transplantation
HTZ	Heterozygosity
ICD	Implantable cardioverter-defibrillator
Ig	Immunoglobulin
IIE	Intron identity element
ISPs	Ion Sphere Particles
IPT	Inositol triphosphate
IVS	Interventricular septum
JMML	Juvenile Myelomonocytic Leukemia
KSS	Kearns–Sayre syndrome
LAFB	Left Anterior Fascicular Block
LBBB	Left Bundle Branch Block
LGMD	Muscular Dystrophy, Limb
LQT	Long QT syndrome
LV	Left Ventricle
LVDD	left ventricular diastolic dysfunction
LVEF	Left Ventricular Ejection Fraction
LVH	Left ventricular hypertrophy
LVM	Left Ventricular Mass
LVNC	Left Ventricular Noncompaction
LVOT	Left ventricular outflow tract
LVOTO	Left ventricular outflow tract obstruction
MADA	Mandibulo acral dysplasia
MAF	Minor Allele Frequency
MAPK	Mitogen-activated protein kinase
MARPs	Muscle Ankyrin Repeat Proteins

MELAS	Mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes
METCDS	Metachondromatosis
MHC	Myosin Heavy Chain
MFM	Myopathy, myofibrillar
MPD1	Myopathy, distal
MPDT	Myopathy, distal Tateyama Type
mtDNA	Mitochondrial DNA
MURF	Muscle specific ring finger protein
MWT	Maximal Wall Thickness
Myct	Myectomy
NE	Not evaluated
NGS	Next Generation Sequencing
nPath	Non-Pathogenic
NO	Nitric oxide
NS	Noonan syndrome
NS1	Noonan syndrome 1
nsSNVs	non-synonimus single nucleotide variants
NSVT	Non-Sustained Ventricular Tachycardia
NYHA	New York Heart Association functional class
PAS	Periodic Acid–Schiff-positive granules
Path	Pathogenic
PCR	Polymerase Chain Reaction
PFHB1A	Progressive familial heart block, type Ia
Pi	Phosphate
PMK	Pacemaker
PnPath	probably-non-pathogenic
PPath	probably-pathogenic
PPKS2	Keratosis Palmoplantaris Striata II
PSVT	Paroxysmal Supraventricular Tachycardia
PT	Patients
RBBB	Right Bundle Branch Block
RCM	Restrictive cardiomyopathy
RMD	Rippling muscle disease
RMSD	Root-mean-square deviation
RV	Right Ventricle
SA	Sinus Arrest
SAM	Systolic anterior movement
sANK-1	Small ankyrin-1
SAR	Supraventricular Arrhythmias
SCD	Sudden Cardiac Death
SCPNK	Scapuloperonal syndrome, neurogenic kaeser type
SFWHS	Skin fragility-woolly hair syndrome
SPMM	Scapuloperoneal syndrome, myopathic type
SR	Sarcoplasmic reticulum
SSS	Sick sinus syndrome, autosomal recessive
Sync/Presync	Syncope/Presyncope
TDI	Doppler imaging

Thor pn	Thoracic pain
TM	Transmembrane region
TS	Target Sequencing
UNK	Unknown
UTR	Untraslated region
VF1	Ventricular fibrillation during myocardial infarction, susceptibility to
VO_2	Ventricular maximal oxygen uptake
VT	Ventricular tachycardia
VUS	Variants of uncertain significance
WPW	Wolff-Parkinson-White Syndrome

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Research paper

Molecular analysis of sarcomeric and non-sarcomeric genes in patients with hypertrophic cardiomyopathy

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ABSTRACT

Background: Hypertrophic cardiomyopathy (HCM) is a common genetic heart disorder characterized by unexplained left ventricle hypertrophy associated with non-dilated ventricular chambers. Several genes encoding heart sarcomeric proteins have been associated to HCM, but a small proportion of HCM patients harbor alterations in other non-sarcomeric *loci*. The variable expression of HCM seems influenced by genetic modifier factors and new sequencing technologies are redefining the understanding of genotype–phenotype relationships, even if the interpretations of the numerous identified variants pose several challenges.
Methods and results: We investigated 62 sarcomeric and non-sarcomeric genes in 41 HCM cases and in 3 HCM-related disorders patients. We employed an integrated approach that combines multiple tools for the prediction, annotation and visualization of functional variants. Genotype–phenotype correlations were carried out for inspecting the involvement of each gene in age onset and clinical variability of HCM. The 80% of the non-syndromic patients showed at least one rare non-synonymous variant (nsSNV) and among them, 58% carried alterations in sarcomeric *loci*, 14% in desmosomal and 7% in other non-sarcomeric ones without any sarcomere change. Statistical analyses revealed an inverse correlation between the number of nsSNVs and age at onset, and a relationship between the clinical variability and number and type of variants.
Conclusions: Our results extend the mutational spectrum of HCM and contribute in defining the molecular pathogenesis and inheritance pattern(s) of this condition. Besides, we delineate a specific procedure for the identification of the most likely pathogenetic variants for a next generation sequencing approach embodied in a clinical context.

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Data article

Prediction and visualization data for the interpretation of sarcomeric and non-sarcomeric DNA variants found in patients with hypertrophic cardiomyopathy

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ABSTRACT

Genomic technologies are redefining the understanding of genotype–phenotype relationships and over the past decade, many bioinformatics algorithms have been developed to predict functional consequences of single nucleotide variants. This article presents the data from a comprehensive computational workflow adopted to assess the biomedical impact of the DNA variants resulting from the experimental study "Molecular analysis of sarcomeric and non-sarcomeric genes in patients with hypertrophic cardiomyopathy" (Bottillo et al., 2016) [1]. Several different independently methods were employed to predict the functional consequences of alleles that result in amino acid substitutions, to study the effect of some DNA variants over the splicing process and

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Cardiovascular Pathology

Clinical Case Report

A novel *LAMP2* mutation associated with severe cardiac hypertrophy and microvascular remodeling in a female with Danon disease: a case report and literature review

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ABSTRACT

Background: Danon disease (DD) is a rare disorder characterized by cardiomyopathy, intellectual disability, and proximal myopathy. It is caused by mutations in the *LAMP2* gene on X chromosome. Female patients most often present with late-onset cardiomyopathy and slow disease progression, but early-onset cases with unfavorable prognosis have been reported.
Case report: We describe the clinical, pathological, and molecular features of a novel *LAMP2* c.453delT mutation in a female patient with severe hypertrophic cardiomyopathy, Wolff Parkinson White (WPW) syndrome and rapid progression to heart failure, requiring heart transplant. Immunohistochemical analysis of *LAMP2* in the explanted heart revealed a mosaic pattern of distribution, with discrete clusters of either stained or unstained cardiac myocytes, the latter being more frequent in the septum. These findings paralleled X chromosome inactivation within the myocardium. Interestingly, multiple foci of microscarring were found on histology in the Left Ventricle (LV) free wall and septum, in a close spatial relationship with remodeling and severe stenosis of intramural coronary arterioles.
Conclusions: Our findings suggest that several features may contribute to the early and severe cardiac phenotype in female DD patients. The type of mutation may account for the early disease onset, while both the inhomogeneous distribution of *LAMP2* loss and the presence of microvascular remodeling may be determinant in the rapid progression to heart failure.

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