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Confronting the archipelago of primary myocardial diseases:

from the comprehension of genetic basis, molecular mechanisms and clinical correlates to the development of novel therapeutic approaches.

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To Bianca,
my little lovely niece.
She lights up my heart every
time I see her playing and smiling.
She has been a true blessing to our family.

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1. Introduction: Tales of the Unexpected

"We hear only those questions for which

we are in a position to find answers"

Friedrich Nietzsche

1.1 Brief history and contemporary classification of cardiomyopathies.

It is somewhat of a paradox that cardiologists invest most of their time treating conditions that only secondarily affect the heart muscle, due to coronary disease, hypertension or the valvular abnormalities. Conversely, substantially less time and resources are devoted to the large and heterogeneous family of diseases originated primarily from the myocardium – *the cardiomyopathies*. Although less prevalent than the previously quoted conditions, cardiomyopathies have a considerable impact on the community, with a 3% estimated prevalence worldwide, generating mortality and morbidity preferentially in the young. Furthermore, they represent valuable paradigms allowing translational investigation of the normal and abnormal functions of the myocardium, creating opportunities for the development of novel treatment with broad implications for all kinds of cardiac patients. Therefore, increased awareness, investments and research efforts in this field are highly desirable.

In its present definition, the term “cardiomyopathy” refers to a myocardial disorder, often genetic in nature, in which the heart muscle is structurally and functionally abnormal in the absence of coronary artery disease, hypertension, valvular or congenital heart disease sufficient to cause the observed myocardial abnormality. This term was first used in 1957 by Brigden, who described a group of uncommon, non-coronary myocardial diseases [1]. In 1961 Goodwin defined cardiomyopathies as “myocardial diseases of unknown cause” [2]. He described three different entities, namely “dilated, hypertrophic and restrictive”, terms which are

still in use today. However, only in the 70s and 80s, following the advent of non-invasive imaging, such as M-mode and 2D echocardiography, the true frequency and spectrum and of cardiomyopathies began to be recognized. In an attempt to provide a practical framework for clinicians involved in the care of these patients, the first classification of cardiomyopathies was published in 1980, by the World Health Organization (WHO) and International Society and Federation of Cardiology (ISFC), and included the three entities proposed by Goodwin [3]. The definition of "myocardial diseases of unknown cause" was maintained to define cardiomyopathies, as opposed to "specific heart muscle diseases", comprising heart diseases with similar phenotypes, but due to an identifiable cause.

In the last 30 years, progress in imaging techniques and intensive genetic investigation have produced major advancements in our understanding of the causes and manifestations of cardiomyopathies [4,5], and new nosologic entities have been described. This led to the new revision of the classification, carried out in 1996 by the WHO and ISFC [6]. Representing a major advancement, both "*arrhythmogenic right ventricular dysplasia"* (with the inappropriate term "dysplasia" later changed to "cardiomyopathy") and a group of "*unclassified cardiomyopathies*'', defined as "those that do not fit in any group", were added to the three original subgroups. The definition of cardiomyopathy was changed to "diseases of the myocardium associated with myocardial dysfunction". Moreover, three additional subgroups termed "hypertensive", "valvular" and "ischemic" cardiomyopathies were - confusingly - added to the group of "specific heart muscle diseases" in order to resolve a terminology controversy between US and European experts [6]. These were defined as cardiac conditions characterized by the

presence of hypertension, coronary or valvular disease, in a degree that would not explain the magnitude of LV dysfunction observed. Nevertheless, a substantial difference in terminology persisted on the two sides of the Atlantic, reflecting the refusal of these fine distinctions by US experts [7].

In 2006, an American Historical Association (AHA) panel of experts published a scientific statement on the "Contemporary classification and definitions of Cardiomyopathies" [7]. They proposed a novel approach, by which the etiology, rather than the phenotype, was used as the main criterion. "Primary" cardiomyopathies were defined as those "involving only the heart", as opposed to the "secondary". Primary cardiomyopathies for the first time also included "ion channel diseases" and were differentiated in three subgroups based on their etiology as "genetic, mixed and acquired" [Figure 1.1-1].

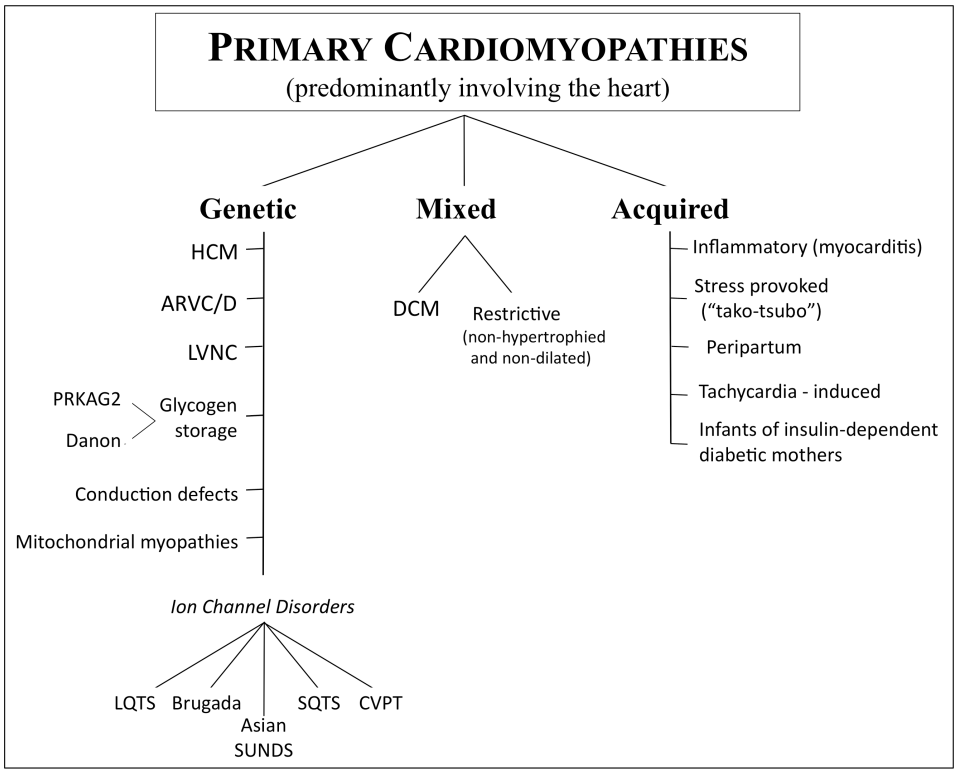


Figure 1.1-1: American Heart Association classification of the cardiomyopathies (2006)

Of note, the term “primary” was used to describe diseases in which the heart is the sole or predominantly involved organ, while “secondary” described diseases in which myocardial dysfunction is part of a systemic disorder [5]. However, the challenge of distinguishing primary and secondary disorders is illustrated by the fact that many of the diseases classified as primary cardiomyopathies can be associated with major extra-cardiac manifestations; conversely, pathology in many of the diseases classed as secondary cardiomyopathies can predominantly (or exclusively) involve the heart.

The radical shift from a phenotypic to an etiological classification, as well as the inclusion of ion channel diseases among cardiomyopathies, although proposed for research rather than clinical purposes, sparked a passionate transatlantic debate, culminating in a thorough re-visitation of the original 1995 classification by the European Society of Cardiology (ESC) Working Group on Myocardial and Pericardial diseases, in 2008 [8]. Intrinsically faithful to the concept of classifying cardiomyopathies based on phenotype, the 2008 European classification maintained each of the time-honoured categories including dilated, hypertrophic, restrictive and arrhythmogenic right ventricular cardiomyopathy. The confusing “hypertensive'', “valvular'' and “ischemic'' categories were removed. In the “unclassified” subgroup, *“Left Ventricular Non-compaction”* and *“Tako-Tsubo”* cardiomyopathy made their official debut [Figure 1.1-2]. Conversely, ion channel diseases were excluded, despite their genetic nature, in view of their lack of a structural cardiac phenotype. Each cardiomyopathy subtype was subdivided in a familial and non-familial subset, and, to replace the pre-genetic era concept of “unknown etiology”, a list of potential genetic and non-genetic causes was provided [Tables 1 to 5].

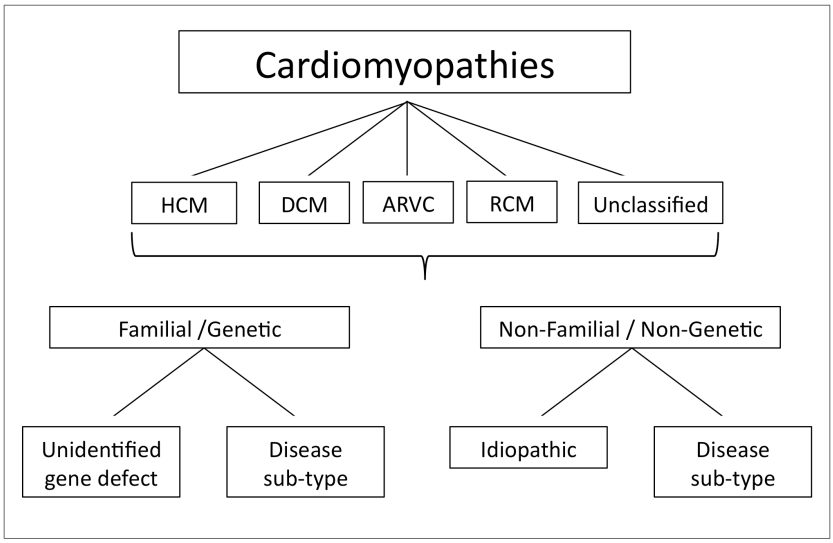


Figure 1.1-2: ESC classification of the cardiomyopathies (2008)

Table 1 to table 5: Genetic and non-genetic causes of cardiomyopathies

Table 1. Hypertrophic cardiomyopathy.

FAMILIAL	Unknown gene
	Sarcomeric protein disease ß myosin heavy chain, Cardiac myosin binding protein C Cardiac troponin I, T and C, a-tropomyosin, Essential myosin light chain, Regulatory myosin light chain, Cardiac actin, a- myosin heavy chain, Titin
	Glycogen storage diseases (e.g. GSD II (Pompe's disease); GSD III (Forbes' disease), AMP kinase (WPW, HCM, conduction disease)
	Lysosomal storage diseases (e.g. Anderson-Fabry disease, Hurler's syndrome)
	Disorders of Fatty Acid Metabolism Carnitine, Phosphorylase B kinase deficiency
	Mitochondrial cytopathies (e.g. MELAS, MERFF, LHON)
	Syndromic HCM Noonan's syndrome, LEOPARD syndrome, Friedreich's ataxia, Beckwith-Wiedermann syndrome; Swyer's syndrome (pure gonadal dysgenesis)
	Other: Muscle LIM protein Phospholamban promoter Familial Amyloid
NON-FAMILIAL	
	Obesity; Infants of diabetic mothers; Athletic training; Amyloid (AL / prealbumin)

Table 2. Dilated cardiomyopathy.

FAMILIAL, unknown gene
- **Sarcomeric protein mutations** (see HCM)
- **Z band :** Cypher/Zasp, Muscle LIM protein, TCAP
- **Cytoskeletal genes**: Dystrophin, Desmin, Metavinculin, Sarcoglycan complex, CRYAB, Epicardin
- **Nuclear membrane**: Lamin A/C, Emerin
- **Mildly dilated CM**
- **Intercalated disc protein mutations** (see ARVC)
- **Mitochondrial cytopathy**

NON FAMILIAL
- **Myocarditis** (infective/toxic/immune)
- **Kawasaki disease**
- **Eosinophilic** (Churg Strauss syndrome)
- **Viral persistence**
- **Drugs, Pregnancy, Endocrine**
- **Nutritional:** thiamine, carnitine, selenium, hypophosphataemia, hypocalcemia.
- **Alcohol**
- **Tachycardiomyopathy**

Table 3. Restrictive cardiomyopathy.

FAMILIAL, unknown gene
- **Sarcomeric protein mutations:** Troponin I (RCM +/− HCM), Essential myosin light chain
- **Familial Amyloidosis** Transthyretin (RCM + neuropathy)
  Apolipoprotein (RCM + nephropathy)
- **Desminopathy**
- **Pseuxanthoma elasticum**
- **Haemochromatosis**
- **Anderson-Fabry disease**
- **Glycogen storage disease**
- **Endomyocardial fibrosis (Familial)** (Fusion FIP1-like-1 / PDGFRA genes)

NON FAMILIAL
- **Amyloid (AL/prealbumin)**
- **Scleroderma**
- **Endomyocardial fibrosis**
  Hypereosinophilic syndrome, Idiopathic chromosomal cause
- **Drugs**: serotonin, methysergide, ergotamine, mercurial agents, busulfan, anthracyclines
- **Carcinoid heart disease, Metastatic cancers, Radiation**

Table 4. Arrhythmogenic right ventricular cardiomyopathy.

FAMILIAL, unknown gene
- **Intercalated disc protein mutations**: Plakoglobin, Desmoplakin, Plakophilin 2, Desmoglein 2, Desmocollin 2
- **Cardiac ryanodine receptor** (RyR2)
- **Transforming growth factor-β3** (TGFβ3)

NON FAMILIAL
- Inflammation?

Table 5. Unclassified cardiomyopathies.

FAMILIAL unknown gene
- **Left ventricular non-compaction:**
  - Barth Syndrome
  - Lamin A/C
  - ZASP
  - a-dystrobrevin

NON FAMILIAL
- **Takotsubo cardiomyopathy**

The precise identification of the disease etiology has obvious clinical implications, by virtue of its direct impact to totally different management. For example, amyloidosis, Anderson Fabry diseases and glycogen storage diseases may be diagnosed as hypertrophic cardiomyopathy (HCM); yet their treatment varies widely. Of note, the inclusion of amyloidosis in this classification was widely debated [9]. Substantial doubts also regarded Takotsubo, a disease that is generally transient, has no proven inherited cause, and appear related to regional myocardial hypoperfusion rather than to heart muscle abnormalities. Ultimately, both were included as this was felt to be conceptually useful in clinical practice.

To follow is a brief overlook of the major cardiomyopathies subtypes, based on contemporary definitions.

1.1.1 Hypertrophic Cardiomyopathy

HCM is a genetic disease characterized by unexplained LV hypertrophy, associated with non-dilated ventricular chambers, in the absence of another cardiac or systemic disease capable of producing that degree of hypertrophy [Figure 1.1-3]. HCM is diagnosed by a maximal LV wall thickness greater than 15 mm, based on echocardiography (Echo) or cardiac magnetic resonance (CMR) [10]. This value is lowered to 13-14 mm, when family members are screened. In children, a wall thickness greater than 2 standard deviations (SD) for age, sex or body size is considered diagnostic.

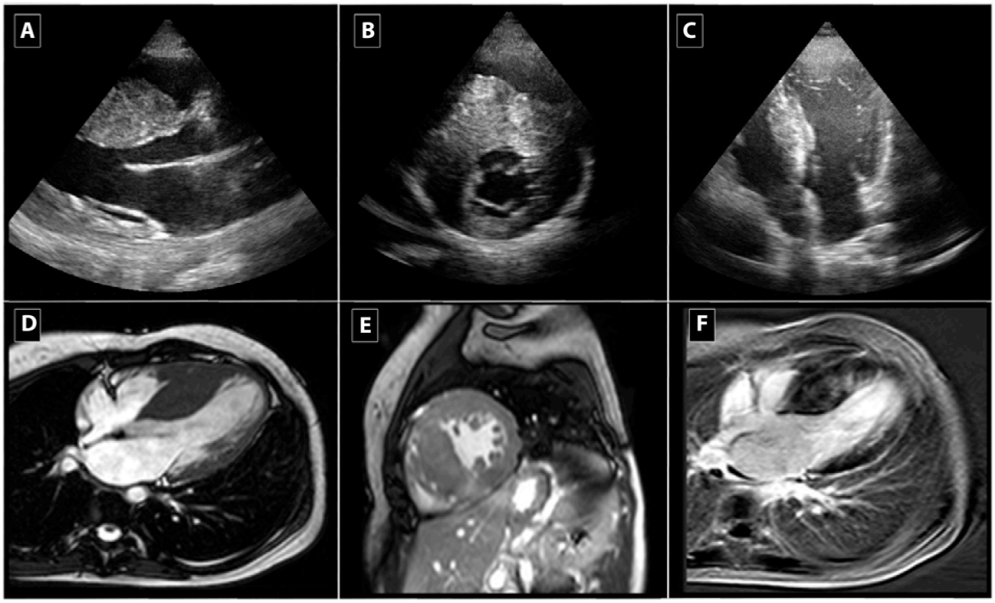


Figure 1.1-3: Figure 3. Hypertrophic cardiomyopathy.

Echocardiographic and cardiac magnetic resonance images from a 17-year old female patient with HCM. Parasternal long and short axis views show severe LV thickness values (max LV wall thickness 31 mm), with redundant mitral leaflets (panels A, B and D) and small cavity size. Apical 4 chambers views show massive hypertrophy of the septum and the antero-lateral wall (panels C and E). Images of late gadolinium enhancement showing limited and nontransmural area of fibrosis of the IVS (panel F: black arrow).

The distribution of hypertrophy is usually asymmetric and sometimes confined to one or two LV segments. As a consequence, LV mass (measured by CMR) can be within the normal range. LV outflow tract obstruction is an important feature of HCM, and may be demonstrated in up to 70% patients [11]. Overall, the clinical course of patients with HCM is relatively benign, with an annual mortality rate of about 1%. Contrary to prior perceptions, the risk of sudden cardiac death is relatively low [12], although still a major concern in young individuals and athletes. Furthermore, about half of patients show some degree of disease progression and functional limitation, with a small but significant subset of about 5% developing the so-called end-stage HCM. Family

screenings, following the introduction of genetic testing, has led to the identification of genotype-positive/phenotype-negative individuals, a novel category within the HCM spectrum, characterized by absence of LV hypertrophy, assessed by ECG and ECHO [10].

Sarcomeric gene mutations, often private, are the most frequent cause of HCM, accounting for approximately 30-65% of probands in different cohorts [13]. In the remaining subset the genetic substrate is unknown. Furthermore, a small proportion of patients with the HCM phenotype are affected by cardiofacial syndromes (e.g. Noonan, LEOPARD, Costello), neuromuscular diseases (e.g. Frederich's ataxia), mitochondrial diseases [14], metabolic disorders of lysosomal storage diseases (i.e. Fabry, Pompe, Danon) [15]. These rare conditions sometimes exhibit an X-linked rather than the autosomal pattern of inheritance, usually observed in HCM [Table 1].

1.1.2 Dilated cardiomyopathy

DCM is characterized by LV dilatation and global systolic dysfunction (EF < 50%), in the absence of coronary artery disease or other identifiable causes (such as systemic hypertension, valve disease, drugs, inflammatory heart diseases) capable of causing that magnitude of impairment [Table 2]. In familial DCM, screening of first-degree relatives will identify the disease in up to 50% [16]. As for many other cardiomyopathies, the prevalence of DCM is underestimated, because many patients may have a subclinical form of the disease, which may be difficult to diagnose for the lack of symptoms. Familial and sporadic forms of DCM have similar morphological manifestation and clinical course [Figure 1.1-4].

However some gene mutations, such as Lamin A/C seem to carry a more adverse outcome, in particular for sudden death [17-19]. DCM is a progressive disease, with a prognosis that, although improved in the last decades, is usually poor due to heart failure, atrial and ventricular arrhythmias, stroke and sudden death [20]. In patients with refractory heart failure, heart transplant represent the final option. The low yield of genetic testing for DCM (i.e. 30%) limits its clinical use and it is related to the large number of potentially disease-causing genes. Furthermore, genetic mutations are usually private and the interpretation of the analysis results may be difficult [16]. However, the advent of new sequencing technologies is probably going to change this paradox in the near future (see chapter 2).

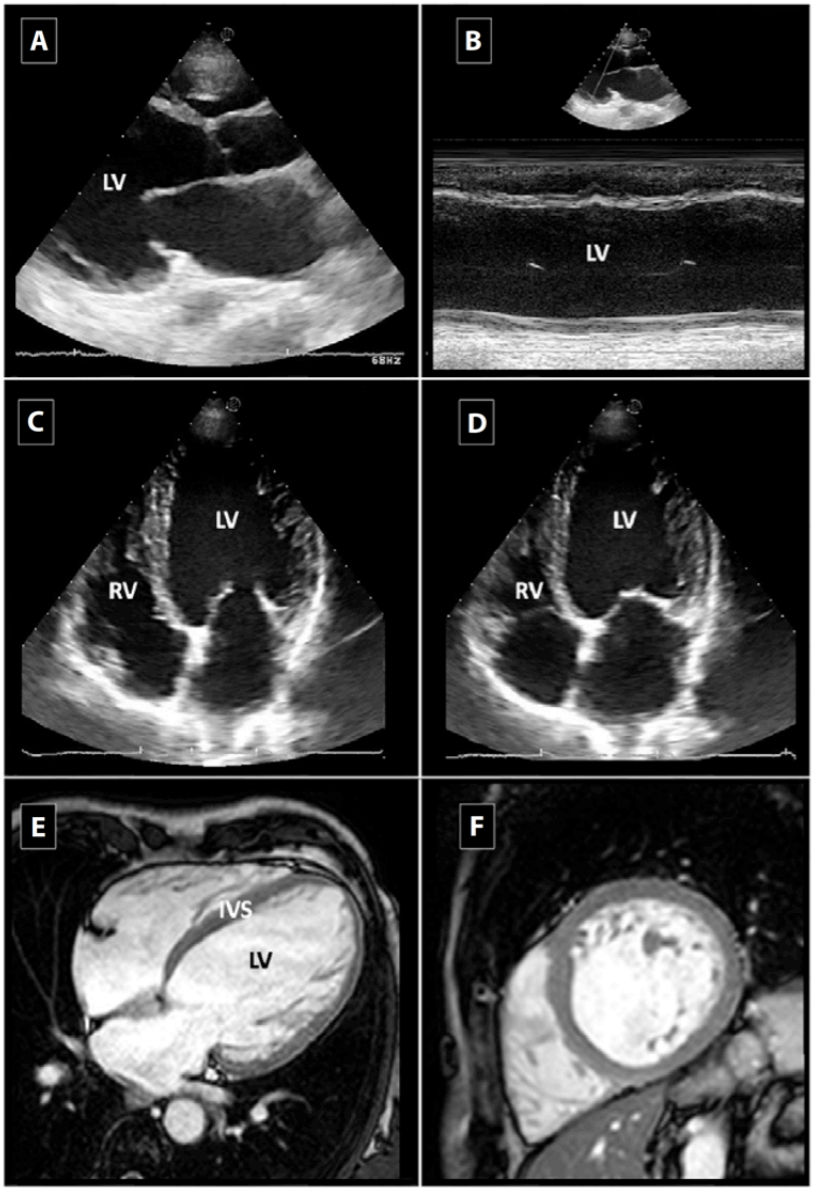


Figure 1.1-4: Dilated cardiomyopathy

Echocardiographic and cardiac magnetic resonance images from a 57-year old female patient with DCM and normal coronary angiogram. Parasternal long axis view and CMR images show dilated LV (panels A-B and E-F), with severe systolic dysfunction – EF = 21%; (panel C = diastole, panel D = systole). *Abbreviations*: LV = left ventricle, RV = right ventricle IVS = inter-ventricular septum.

1.1.3 Restrictive cardiomyopathy

RCM is defined by the presence of a restrictive LV physiology, with normal or more often reduced diastolic/systolic volumes, normal wall thickness and systolic function, marked diastolic flow impairment and biatrial dilatation. RCMs are rather uncommon, although their prevalence is still unknown. Either Amyloid Light-chain (AL) amyloidosis or amyloidosis due to transthyretin gene mutations with heart involvement, often cause RCM [Table 3] [9]. A striking subtype of disease with restrictive physiology, endomyocardial fibrosis, endemic in areas of the African continent, has an unknown etiology and very poor prognosis [21]. Moreover a “restrictive phenotype” may be part of the clinical spectrum of end-stage HCM [22], and may occasionally originate as a primary, non HCM-related phenotype from sarcomere gene mutations (generally in the thin filament protein coding genes – *see chapter 2*). RCM is usually associated with severe functional limitation, mainly related to the extreme diastolic dysfunction, with reduced diastolic filling and stroke volume, and a poor prognosis [23].

1.1.4 Arrhythmogenic right ventricular cardiomyopathy

ARVC is characterized by fibro-fatty replacement of the right ventricular myocardium and ventricular arrhythmias [24]. In the most common right-dominant form, structural changes may be absent or confined to a localized region of the right ventricle (inflow and outflow tract, right ventricular apex, known as the `triangle of dysplasia') at an early stage [Figure 1.1-5]. Progression to more diffuse right ventricular disease and LV involvement (typically affecting the posterior lateral wall), associated with ventricular systolic dysfunction, is common at later stages [25]. Ventricular arrhythmias are the clinical hallmark of the disease, but

atrial fibrillation may also occur. The diagnosis of ARVC is often challenging for the cardiologist, in particular during the early "concealed phase", when individuals are still asymptomatic and structural abnormalities are subtle. Predominant LV disease has also been recognized in a small subset of patients of about 5%. New diagnostic criteria with higher sensitivity and specificity have recently been published [24,26]. ARVC is generally a familial disease with autosomal dominant inheritance but it may be recessive when associated with woolly hair and palmopalmar hyperkeratosis (eg, Naxos disease, Carvajal syndrome). Mutations in desmosomal and non-desmosomal genes have been identified, but interpretation of their pathogenicity is often challenging in the affected individual [Table 4].

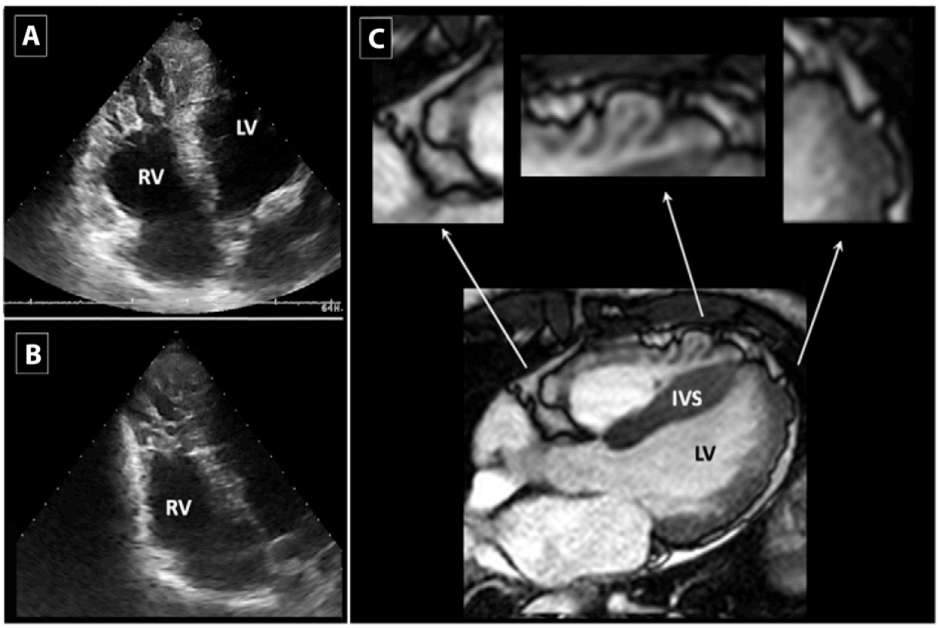


Figure 1.1-5: Arrhythmogenic cardiomyopathy

38 year-old female with diagnosis of ARVC, resuscitated from out-of-hospital cardiac arrest. She has family history of ARVC (mother) and sudden death (brother, 28 year old). CMR images show clearly wall aneurysms within the so-called ``triangle of dysplasia'' (panel AC, white arrows: evident systolic bulging in infundibular, apical, and subtricuspid regions of the RV). She also had LV involvement (apex).

Abbreviations: LV = left ventricle, RV = right ventricle, IVS= interventricular septum.

1.1.5 Unclassified cardiomyopathies

Isolated LV non-compaction (LVNC) is characterized by prominent LV trabeculae and deep inter-trabecular recesses, that can be associated with LV dilatation and systolic dysfunction [Figure 1.1-6].

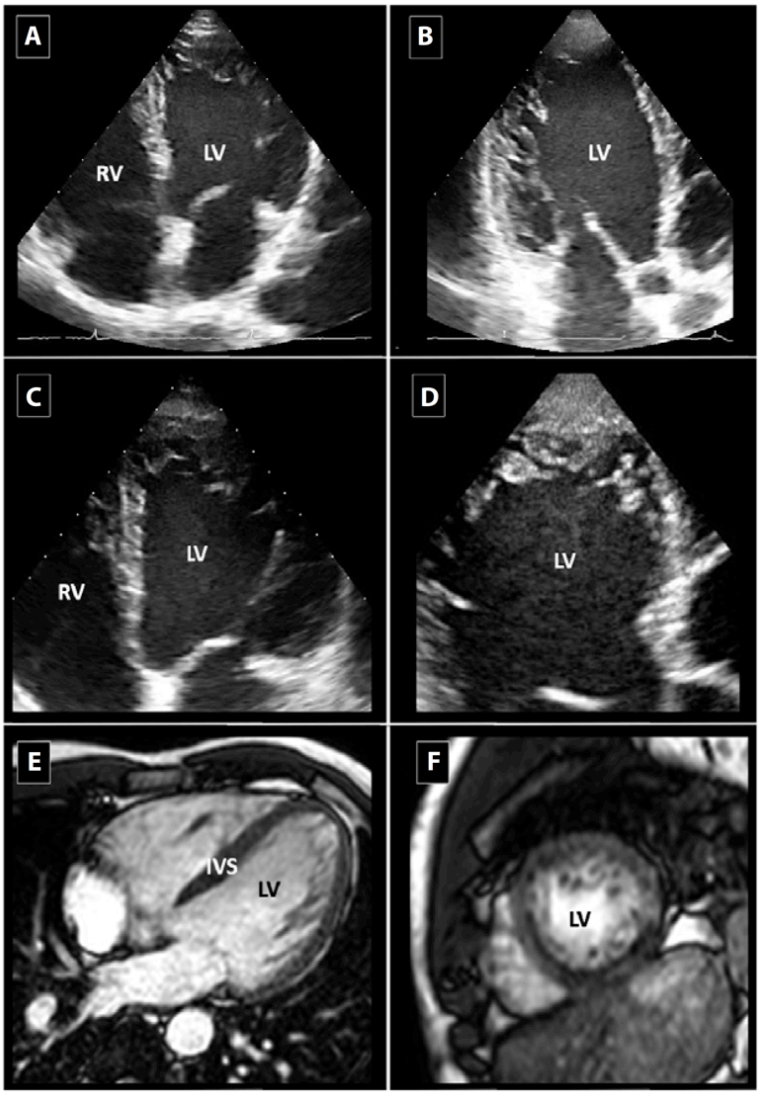


Figure 1.1-6: Left ventricular non compaction

Isolated left ventricular non-compaction in a 45 year-old male, with mild systolic dysfunction (EF 48%), ventricular arrhythmias and normal LV diameters. Multiple trabeculations and recesses are evident, particularly

in the apex and the free wall of the LV (panels A and C: apical 4 chambers view; panels B and D: apical 3 chambers view). CMR confirmed the diagnosis (panels E-F). *Abbreviations*: LV= left ventricle, RV = right ventricle IVS = inter-ventricular septum.

LVNC is familial, with 25% of asymptomatic first-degree relatives having some echocardiographic abnormalities [Table 5]. Of note, this rather mysterious disease shows substantial phenotypic overlap with other cardiomyopathies (in particular HCM and DCM, which often exhibit limited areas of non-compaction in the left ventricle), as well as a common genetic substrate [8]. Furthermore, LVNC may be associated with congenital cardiac disorders (such as Ebstein's anomaly or complex cyanotic heart disease) and some neuromuscular diseases. Therefore, it is still debated whether isolated LVNC should be considered a separate clinical and genetic entity, or a morphological trait shared by many distinct cardiomyopathies. As a result of the difficult comprehension of this clinical entity, the real prevalence of LVNC and its outcome remain largely unknown.

Takotsubo cardiomyopathy, also known as LV apical ballooning or stress-induced cardiomyopathy, is characterized by transient regional systolic dysfunction involving the apex and/or mid-ventricle in the absence of obstructive coronary artery disease on angiogram [8]. The condition is reported all over the world, and most reported cases occur in post-menopausal women following physical or psychological stress, but it has been described also in patients with intracranial haemorrhage or other acute cerebral accidents (so-called “neurogenic myocardial stunning”). Typically, takotsubo cardiomyopathy has a sudden onset, with chest pain, diffuse T-wave inversion and mild cardiac enzyme elevation. Symptoms are often preceded by emotional or physical stress. If the patient survives the acute phase of disease, the prognosis is almost

invariably favourable, with a normalization of LV function over a period of days to weeks; recurrence is possible, but rare.

1.1.6 A never-ending story

As more families with cardiomyopathies are genotyped, and new diseases are being described, the paradigm “one gene, one disease” appears no longer sustainable. The same mutation can be expressed at a different age and give rise to hugely different phenotypes within the same family. Different phenotype patterns may originate from the same genetic substrates, in a spectrum encompassing HCM, DCM, RCM and LVNC (all associated with sarcomere genes), or ARVC/DCM (associated with desmosomal genes). Such heterogeneity is thought to derive from the interaction between one or more genetic mutations, modifier genes and environmental factors [10]. When genetic analysis is performed in candidate genes, the probability of identifying the pathogenic gene mutation is in the range of 40 60%, for patients with HCM, with approximately 5% of complex mutations [11,12]. Results for dilated cardiomyopathy (DCM), restrictive cardiomyopathy (RCM) and isolated LV non-compaction are considerably less rewarding [10], although the advent of next generation, genome-wide techniques may increase the yield substantially, as recent data on titin in DCM suggests [27]. The recent introduction of next generation sequencing has started what promises to be a revolution in molecular diagnostics, allowing rapid and affordable testing of hundreds of genes, or even whole genomes. As an example, a wide range of truncating gene mutations encoding Titin, a very large cytoskeleton gene which could not be assessed by traditional sequencing techniques, has recently been discovered to represent a prevalent cause of familial DCM, up to 25% [27]. In the near future, the

list of causative genes will therefore likely require an update. The focus for researchers will necessarily shift from analyzing single mutations in candidate genes, to interpreting the hundreds of variants of unknown significance in putative causative as well as modifier genes, requiring entirely new skills and significant interaction with biophysicists and computer scientists.

At present, and in the foreseeable future, however, clinical classifications of cardiomyopathies based on clinical presentation and morphological criteria represent an important tool for clinicians involved with these complex diseases. While calling for constant improvement and update in the light of advances provided by imaging, genetics and basic science, individual patient phenotypes continue to represent the core of any classification in clinical medicine, something that has not changed with time.

1.2 A thousand conditions with a lot in common. Translational overview supporting a comprehensive approach to primary diseases of the myocardium.

The pathophysiology of cardiomyopathies is extraordinarily complex and encompasses a constellation of different mechanisms, most of which at present unresolved. Even when the disease model is narrowly defined and recognizes a well-defined etiology, such as a gene mutation, the processes leading to the phenotypic manifestations are largely beyond our current understanding. For example, early hopes of establishing strict genotype-phenotype correlations in HCM, probably the most extensively studied disease in this group, have definitely been abandoned. Even when the link between mutations and myocardial damage is relatively understood, as in Anderson Fabry disease, the reasons of clinical heterogeneity observed even among individuals from the same family remain obscure. It is now well established that the same genes can give rise to radically different phenotypic and clinical manifestations and – conversely – that the same phenotype can be caused by mutations in different genes. In other words, gene mutations are necessary but not sufficient to cause clinical phenotypes, and what comes in between is at least as important as the etiology itself. In what may seem like an inextricable labyrinth, however, a number of general mechanisms seem to represent common pathophysiologic determinants of cardiomyopathy phenotypes. While not specifically related to the disease etiology, these features represent shared pathways by which the disease becomes overt and leads to its clinical consequences; in some cases they represent features are common with heart failure due to any cause. Any attempt to comprehensively cover this field goes, is would be too ambitious.

What follows is an overview of some of the most intriguing elements characterizing cardiomyopathy pathophysiology, representing both keys to further understanding of the "core" of heart muscle disease, and potential targets for treatment.

1.2.1 Genetic and post-transcriptional mechanisms

The variable, often age related, penetrance and variable disease expression suggest that the effects of cardiomyopathy-causing mutations are modifiable both by modifier genes and environmental factors [28]. Mutations generally cause single amino-acid substitutions in proteins that become incorporated into the sarcomere and exert their pathological effects by acting as poison peptides that alter normal sarcomere function in a concentration dependent manner. Thus, homozygous mutations, multiple mutations, and compound genotypes (2 or more mutations in multiple genes) often result into earlier presentation and rapid disease progression [29-31]: this has been shown for HCM, ARVC and ion channel disease. An exception to this rule are most MYBPC3 mutations in HCM, that result in insufficient protein production for normal sarcomere function (haploinsufficiency) [32-33]. Haploinsufficiency can be attributed to cell surveillance mechanisms, including nonsense-mediated decay of mRNA transcripts that contain premature termination codons and/or ubiquitin-mediated proteasomal (UPS) degradation of misfolded proteins. It remains to be demonstrated whether impairment of UPS due to excess degradation of mutant proteins may trigger phenotypic onset or contribute to disease progression [34]. In addition, differential levels of activity in these cell surveillance mechanisms may explain individual heterogeneity in phenotype, eg within families with the same genetic mutation. Finally, MicroRNAs (miRNAs) are small conserved RNA

molecules nucleotides which negatively modulate gene expression in animals and plants. MiRNAs are involved in a variety of basic biological processes, for example, cell proliferation and apoptosis and stress responses. A subset of miRNAs are either specifically or highly expressed in cardiac muscle, providing an opportunity to understand how gene expression is controlled by miRNAs at the post-transcriptional level in this muscle type. miR-1, miR-133, miR-206, and miR-208 have been found to be muscle-specific, and thus have been called *myomiRs*; among their functions, they have been shown to regulate cardiac development and differentiation. MiRNAs are thought to play an important role in modulating the development of phenotype in patients with cardiomyopathies. For example, in vivo miR-133 levels are down-modulated in patients with HCM, and other MiRNAs have been implicated in the regulation of cardiac hypertrophy [35]. Therefore, both the initial genetic burden and post-transcriptional mechanisms seem to significantly impact the development of cardiomyopathies.

1.2.2 Abnormal calcium homeostasis

Most models of heart failure, including human disease, are characterized by decreased SR Ca2+- ATPase (SERCA) expression and upregulation of Na+/Ca2+ exchanger (NCX) expression and function. Decreased SERCA activity, by slowing down Ca2+ reuptake to the sarcoplasmic reticulum, allows more Ca2+ to be extruded via the NCX; coupled with the increased NCX expression [36] this results in a net loss of cell Ca2+ and contributes to the reduction of sarcoplasmic reticulum Ca2+ load [37]. Another contributor to the lower Ca2+ content in heart failure is the increased diastolic leakage of Ca2+ from the sarcoplasmic reticulum, which is determined by the hyperphosphorylation of ryanodine

receptors by protein-kinase A and/or Ca2+-Calmodulin dependent protein kinase-II (CaMKII) [38]. CaMKII activity is increased in HF, and CaMKII-dependent phosphorylation of RyR enhances Ca2+ spark frequency and thus spontaneous diastolic sarcoplasmic reticulum Ca2+ leak [39], making it a leading pathway causing contractile dysfunction and arrhythmogenesis in HF. Enhanced NCX function, combined with the higher probability of spontaneous Ca2+ release from the SR, contribute directly to arrhythmogenesis via delayed-afterdepolarizations (DADs). When a large spontaneous Ca2+ release event occurs during diastole giving rise to a generalized Ca2+ wave, part of the released Ca2+ is extruded through the NCX, which generates an inward current that depolarizes the membrane (i.e. a DAD). If large enough, a DAD may reach the threshold for a premature AP, giving rise to a premature activation that can propagate through the myocardium, triggering sustained arrhythmias. Thus, abnormal calcium homeostasis is a main determinant of several manifestations that are common to several cardiomyopathies, including diastolic dysfunction (due to the excess residual cytoplasmic Ca2+ at the end of systole), impaired energetics, myocardial ischemia and arrhythmgenesis. This has been specifically shown in models and human tissue with HCM, for example.

1.2.3 Lack of energetic sustainability

Primary myocardial disease is often characterized by abnormal energy generation and/or consumption. In rare diseases, such as cardiomyopathies associated with mitochondrial disease, this feature is taken to the extreme. However, varying levels of energetic impairment can be found in virtually all models. Notably, this is not always due to insufficient energy generation; in HCM for example, disease causing

mutations are often gain-of-function, and lead to sarcomere energetic inefficiency, due to the excess ATP utilization required to generate isometric tension [40, 41], which may ultimately compromise overall cardiomyocyte energetic balance [42]. Energy deficiency would be expected to reduce the activity of membrane-bound energy-requiring ion transporters, potentially triggering arrhythmias and contributing to diastolic dysfunction, and to decrease contractile reserve. In addition, cardiomyocyte energetic compromise might contribute to generation of pathologic hypertrophy (and potentially adverse remodeling) as a consequence of intracellular energy sensor activation [4]. In addition, residual, force-generating, ATP-consuming acto-myosin interactions during diastole lead to incomplete relaxation and directly contribute to diastolic dysfunction while increasing the energetic compromise of HCM myocytes. Sarcomeres and their Z-disk components are now recognized centers of mechano-sensation, -transmission and -transduction [43, 44]. Cardiac stress leads to mechanical and chemical signals which remodel sarcomeres and either offset or exacerbate the stress. In HCM patients, altered sarcomere mechanics due to faster kinetics of force generation, hypercontractility or incomplete relaxation may trigger hypertrophy and adverse remodeling. That this may represent an important primary mechanism is supported by the HCM-causing role of mutations in genes encoding Z-disk proteins [45]. In the long term, reduced energy production or inefficient utilization may become non-sustainable, and directly contribute to a vicious cycle of worsening cardiomyocyte dysfunction, increased LV wall stress, increased oxygen demand and further energetic mismatch, leading to disease progression and heart failure.

1.2.4 Electrophysiological remodeling

The fact that cardiomyopathies may express an electrophysiological, as well as structural, cellular phenotype has only recently been appreciated. While clinicians have always known the surface ECG manifestations associated with each disease, these have classically been attributed to macroscopic abnormalities of the heart such as disarray, strain, hypertrophy and necrosis. However, it is now evident that some of these manifestations (such as QT prolongation) and even the arrhythmogenic potential associated with primary heart muscle disease may originate at the level of the cardiomyocyte sarcolemma. Among the most prominent abnormalities are alterations of late Na+ current in HF. Increased [Na+]i in HF may be due to increased Na+ influx or decreased Na+ efflux. Early studies suggested that an excess of Na+ influx is the major contributor to Na+ overload and the main source of the increased Na+ entry is the enhanced “late” or “persistent” Na+ current [46, 47]. As explained before, a component of Na+ current with slow or incomplete inactivation can be measured in normal human and animal cardiac myocytes [48]. However, acquired and primary cardiac diseases are commonly characterized by abnormally large INaL. Enhancement of late Na+ current has been identified in HCM, and may contribute to its pathogenesis. In addition, besides LQT3 syndrome, which is directly due to Na+ channel mutations [49], increase INaL was found as a consequence of ankyrin B mutations (LQT4 syndrome) and caveolin-3 mutations (LQT-CAV3) [50, 51], as well as end stage heart failure [52, 53] and following myocardial infarction [54]. Increased Ca2+ -Calmodulin and calmodulin kinase II (CaMKII) activity, both common features of myocardial remodelling in HF, have been shown to increase INaL. Recent evidence identified CaMKII-mediated phosphorylation of cardiac Na+ channels at multiple sites is the main regulator of Na+ channel inactivation. INaL

enhancement is associated with prolonged repolarisation causing a remarkable increase in action potential duration [55, 56]. Atrial potential prolongation leads to reduced repolarisation reserve and therefore increased incidence of early afterdepolarizations (EADs, i.e. premature depolarizations occurring during the plateau phase) and potentially fatal arrhythmias [57]. All these conditions are all characterized by increased susceptibility to perturbations of repolarization (e.g. drugs blocking K+ currents or electrolyte imbalances) and overall increased risk of arrhythmias.

1.2.5 Coronary microvascular dysfunction

By definition, cardiomyopathies are characterized by the absence of acquired or congenital coronary artery disease at the epicardial level. Yet, a quota of myocardial ischemia is virtually always present. This is due to impairment of flow reserve at the microvascular level – a crucial determinant of myocardial perfusion. Coronary microvascular dysfunction has been demonstrated in diseases ranging from HCM, DCM, ARVC to Anderson Fabry disease [58-61]. In most studies, impairment of coronary reserve at this level has been shown to have profound prognostic implications [62]. Therefore, whatever the mechanism(s) leading to microvascular dysfunction, the latter tends to take center stage and determine disease progression and outcome when severe, representing an important (and unfortunately yet unattainable) therapeutic target. Mechanisms may be several: the most common is non-specific, represented by extravascular compression of the small coronary vessels due to increased LV wall tension and fibrosis [63]. However, disease-specific mechanisms exist, such as endothelial

infiltration in storage disease (most notably Anderson-Fabry disease) and microvascular remodeling in HCM.

In HCM patients, microvascular remodeling is a striking and consistent feature which occurs independent of hypertrophy, appears to be genetically regulated [64], and may initiate as early as during development [65]. Abnormalities include marked thickening of the intramural coronary arteriole wall, due to smooth muscle hyperplasia and an abundance of disorganized elastic fibres, causing deformation and irregular narrowing of the vessel lumen: as a consequence, their capacity to vasodilate in response to physiological stimuli is markedly impaired. Other factors contributing to microvascular dysfunction, such as disarray, reduced capillary density and increased extravascular compressive forces, are probably active in the most hypertrophied regions of the myocardium.

1.2.6 Fibrosis

Marked increase in the extracellular matrix is another common theme in cardiomyopathy. Collagen synthesis may be primarily triggered by altered fibroblast function (eg due to paracrine or neuroendocrine influences) or as a reparative phenomenon following myocyte loss [66].

Primary (i.e. non ischemia-mediated) activation of profibrotic pathways is a consistent finding in patients with cardiomyopathies, and has been demonstrated even in the pre-clinical phase [67]. These pathways are associated with variable levels of interstitial fibrosis, the clinical relevance of which remains uncertain. Of note, biomarkers of collagen synthesis and degradation reflect collagen metabolism and correlate with adverse outcomes in hypertension, heart failure, and myocardial infarction. In HCM, studies of the links relating sarcomere

protein mutations to the mechanisms that regulate vascular remodeling and fibrosis are likely a key to our understanding of disease progression. Recent studies have suggested that some phenotypic expressions correlate more with altered function of fibroblasts than with myocyte-related pathology. Animal models of HCM that recapitulate human disease have recently shed light on the earliest cellular and molecular responses to sarcomere-gene mutations [68]. Cardiac transcriptional profiling in young mice in which hypertrophy has not yet developed shows activation of pathways involved in fibrosis and collagen deposition. These studies indicate that a profibrotic milieu is present early in hearts with HCM even when cardiac histologic findings are normal.

Replacement fibrosis is discrete, rather than interstitial, may be transmural and occupy significant proportions of the left and/or right ventricle, can be visualized in vivo as late gadolinium enhancement (LGE) by CMR, and is generally associated with systolic dysfunction [69-76]. LGE is believed to largely reflect a reparative process following microvascular ischemia-mediated damage – i.e. a scar [77].

The deposition of LGE varies according to the specific disease. In HCM, areas of LGE have typical mid-wall localization sparing the subendocardial region [70-76]. CMR late gadolinium enhancement is present in about two-thirds of HCM patients, varying from very limited to large, confluent, infarct-like patches occupying significant proportions of the LV [70, 74]. LGE localizes preferentially to the most hypertrophied regions of the ventricle, often represented by the basal and mid-septum, and are more often found in patients with diffuse and severe hypertrophy. Conversely, LGE is often absent in patients with mild HCM phenotype and limited extension of hypertrophy [70,71]. LGE is inversely related to LV systolic function in HCM patients, and that those individuals who have

reached the end-stage phase of the disease, characterized by overt systolic dysfunction and LV remodelling with progressive wall thinning, constantly exhibit large and often transmural areas of LGE, which may occupy as much as 50% of the whole ventricular mass [78]. Furthermore, preliminary evidence points to LGE areas as a potential substrate of ventricular arrhythmias [69, 72].

In ARVC patients, phenotype, although genetically determined, develops postnatally and is progressive with age, initiated by necrotic cell death, which subsequently triggers an inflammatory response and massive calcification within the myocardium, followed later by injury repair with fibrous tissue replacement. In a Desmoglein 2 mutant mouse model, necrosis always preceded other pathological signs of disease, such as inflammation and calcification, and later on, fibrosis, ventricular dilation, and aneurysm formation. Furthermore, myocyte necrosis originated in the subepicardial myocardium, followed by a wave-front extension toward the endocardium [79]. This wave-front phenomenon of myocardial atrophy is well documented in human ARVC, both by histopathologic examinations and cardiac magnetic resonance studies [80, 81].

Both reactive (interstitial and perivascular) and reparative (replacement) myocardial fibrosis (MF) are hallmarks of DCM [82-84]. In vivo assessment of myocardial fibrosis by CMR in DCM patients have shown a prevalence of significant LGE in almost 50%, with mid-wall distribution and typical sparing of the subendocardium – a useful marker to rule out ischemic dilated cardiomyopathy. Fibrosis is a predictor of outcome and reverse remodeling following HF therapy; of note, it is less prevalent in DCM caused by myocarditis, in which LV dysfunction may be reversible, compared to genetic DCM [85].

1.2.7 Developmental aspects

At birth, patients with genetic cardiomyopathies generally exhibit normal hearts, and the full-fledged phenotype may develop at adolescence or during adult life; pediatric onset is rare but possible. When the phenotype develops, a number of tissues and cell types that do not express the mutated gene are found to actively participate in the disease process, in ways that are hard to explain simply on the basis of secondary, bystander, involvement. To date, the link between the genetic defect such "extended" phenotype remains elusive. Among the possible explanation, the hypothesis of cardiomyopathies as cell lineage diseases has emerged, by which pre-natal mechanisms directly linked to the causal gene defect acts upon multipotent progenitors to influence their commitment and ultimate development [65].

For example, theories explaining the development of hypertrophy in HCM patients fail to address aspects of HCM as diverse as interstitial fibrosis, microvascular remodeling and mitral valve. Specific features, such as direct papillary insertion into the mitral leaflet or myocardial bridging, clearly suggest a developmental defect [86]. Our group has proposed [65] that a common lineage ancestry for these extramyocardial phenotypes can be traced back to the proepicardial organ, originating from the posterior component of the secondary heart field [87]. Early during development, the migration of proepicardial cells over the naked heart tube originates the epicardium. Following a process called epithelial-mesenchymal transformation, apparently pluripotent epicardium-derived cells (EPDCs) subsequently migrate diffusely into the myocardium and differentiate into diverse cell types which give rise to cardiac scaffolding as interstitial fibroblasts, to the coronary vasculature

as smooth muscle cells and adventitial fibroblasts, and to the atrio-ventricular cushion tissue as mesenchymal cells [87]. At the time when EPDC migration occurs from the epicardium into the myocardium, the heart has already begun to contract, and most known HCM-causing mutations, such as those involving the beta-myosin heavy chain and myosin-binding protein C genes, are already expressed in the embryonic heart [87, 88]. Therefore, it is tempting to speculate that an interference with the EPDC migration and differentiation processes, by a putative mechanism of mechanotransduction [89], may account for features as diverse as myocardial disarray, interstitial fibrosis, mitral valve abnormalities and microvascular remodeling [65]. Another explanation for why a mutation in sarcomeric proteins can affect valve development is that during development, EPDCs—rather than becoming hypertrophic like other cardiomyocytes—differentiate or revert into fibroblastic-like cells. If this is true, one would expect increased levels of periostin production in hypertrophic hearts since the hallmark of fibroblastic differentiation is expression of periostin. Consistent with this hypothesis, markedly elevated levels of periostin are indeed expressed in HCM mice [90].

Similar hypotheses may explain the cellular origin of adipocytes in ARVC, which represents an enigma [91]. In the heart, the only cell type known to express desmosomal proteins is the cardiomyocyte, which are terminally differentiated in the adult and hence not candidates to dedifferentiate to adipocytes. However, lineage tracing experiments have shown showed that adipocytes in ARVC originate from second heart field progenitor cells that preferentially differentiate into adipocytes because of suppressed canonical Wnt signaling. Indeed, Wnt/β-catenin signaling is an important switch regulator of myogenesis versus adipogenesis and a differentiation of cardiac progenitor cells. These findings may also explain

the predominant involvement of the right ventricle in ARVC, as that the second heart field progenitors give rise to the right ventricle and its outflow tracts through mechanisms governed by canonical Wnt signaling.

Further understanding of the effects of cardiomyopathy-causing mutations during development, and their protean implication on clinical phenotype, may provide important clues on the essence of genetic cardiac disease. These are difficult studies to perform, and require hard multidisciplinary work. However, the rewards may include a completely new way of conceiving the spectrum of these conditions, as well as novel therapeutic targets.

1.3 Making sense of diversity: finding common answers in clinical practice.

Imagining a disease as a fruit or a planet, there are several levels at which one can intervene with any therapeutic approach. The first and most obvious is to simply scratch the surface and control symptoms. This objective can be achieved in most cardiac patients: however, it is the very least we can do. The second step is to interfere and possibly halt disease progression, thus preventing its consequences on outcome: this can be done in several cardiac conditions, but is definitely harder to achieve [92-93]. Third, we can try to prevent the development of full-blown disease in patients who are predisposed due to acquired risk factors and/or genetic substrate [94]. And fourth, we can address the core of the problem by acting directly on the etiology, removing the actual cause and ultimately cure the patient [95]. Despite extraordinary progress over the last decades, these last two steps have hardly ever been achieved in cardiovascular medicine. As a consequence, it is important to realize that our practice is based on highly sophisticated palliation. What this approach usually does is change a disease into a milder one. For example septal myectomy turns obstructive into non-obstructive HCM, and the ICD can prevent malignant arrhythmias in ARVC or DCM, all highly significant benefits for the patients [96, 97]. Therefore, this kind of very effective palliation is something we should definitely keep on doing, and improving, until a cure becomes available. However, all efforts should be directed at improving the state of things by accumulating new evidence and knowledge [98].

Despite decades of increasing attention and research efforts by the scientific community, treatment strategies for cardiomyopathies remain largely based on a small number of clinical studies, or empirically based on personal experience or extrapolation from other cardiac conditions. As stated in the recent Report of the Working Group of the National Heart, Lung, and Blood Institute on Research Priorities in Hypertrophic Cardiomyopathy [98], "nearly 50 years after the identification of HCM as an autosomal dominant disease, and 20 years after its linkage to sarcomeric protein mutations, we still do not understand the most proximal mechanism(s) that initiates the disorder"; and "treatment recommendations in HCM are based on observational series without prospective randomized controls. While clinical usage provides support that various pharmacologic agents reduce HCM symptoms, no evidence has demonstrated that they alter disease progression or outcomes." In a recent review of original articles, reviews and editorials addressing any pharmacological agent ever used in HCM cohorts, only 45 studies were identified over the last sixty years (i.e. less than 1 per year), enrolling a total of 2,121 HCM patients [95]. Of these, only 5 were randomized, double blind placebo-controlled trials. Remarkably, a comparison of the period 1991-2011 vs. 1971-1990 demonstrated no increase in the number of studies, and only a modest increase in the number of patients enrolled (627 vs. 1,473, patients respectively).

Several reasons - some obvious, other less so - stand behind this state of things. The first lies with the practical challenges inherent in designing trials with cardiomyopathy patients. The epidemiology of these conditions is complex and only partially known, due to issues such as scarce awareness, incomplete penetrance and prevalence of subclinical disease [67]. Despite not being rare, cardiomyopathies are uncommonly

encountered and possibly neglected at community-based cardiac centers and outpatient clinics. Furthermore, even when overt and correctly diagnosed, their clinical spectrum is highly heterogeneous, encompassing different stages that may not be directly comparable. A preventive trial in genotype-positive / phenotype-negative individuals will necessarily enroll subsets that are different from patients with end-stage disease. Each research question should be addressed by targeting the appropriate patient subgroups, with imaginable problems in achieving the desired yield in any given cohort [98].

As highlighted in the previous section, several targets for treatment have been identified, some of which overlap with other cardiac diseases and with heart failure at large.

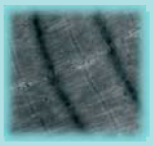
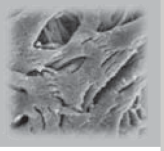
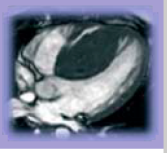
LEVEL OF INTERVENTION	TARGET	GOAL
MOLECULAR 	Abnormal Sarcomere Function Enhanced Ca^{++} Sensitivity Unfavourable EP Remodeling	Improve Contractile Efficiency Inhibit Triggers for LVH Reduce Arrhythmogesis
CELLULAR 	Deranged Energy Homeostasis Disturbed Biomechanical Stress Sensing Abnormal Ca^{++} Handling	Prevent Energy Depletion Prevent Hypertrophy/Apoptosis Control Collagen Synthesis
ORGAN 	Microvascular remodelling Myocardial Fibrosis Diastolic Dysfunction Reentry Arrhythmias	Reduce Myocardial Ischemia Preserve Systolic Function Improve Diastole Prevent Arrhythmias

Figure 1.3-1: Therapeutic targets and goals in cardiomyopathies

For example, progressive interstitial cardiac fibrosis, resulting from non–myocyte (e.g., fibroblast)-mediated activation of transforming growth factorβ signaling, is a common feature of most cardiomyopathies [4]. The finding that preemptive angiotensin II type 1–receptor inhibition prevented myocardial fibrosis in a mouse model of cardiomyopathy, as well as encouraging results from a small clinical study, supports further investigation of this approach [28].

Furthermore, interventions aimed at normalizing energy homeostasis represent a viable approach, as shown by a recent study on perhexiline, a metabolic modulator which inhibits the metabolism of free fatty acids and enhances carbohydrate utilization by the cardiomyocyte. In a randomized, double-blind placebo-controlled trial, perhexiline has recently shown the capacity to improve the energetic profile of the LV, resulting in improved diastolic function and exercise capacity in HCM patients [99]. HCM cardiomyocytes exhibit well-established abnormalities in intracellular calcium handling, contributing to excessive energy expenditure and enhanced arrhythmogenesis, that are largelydue to enhanced membrane late sodium current [100]. Such defect may be selectively and dramatically reversed in vitro by ranolazine. Following the demonstration of its beneficial effects on HCM cardiomyocytes, a multicenter, double blind, placebo-controlled pilot study is currently underway in Europe, to test the efficacy of ranolazine on exercise tolerance and diastolic function in symptomatic HCM patients (RESTYLE-HCM; EUDRA-CT 2011-004507-20). Besides the specific merits of ranolazine, similar examples of translational approach identify a fundamental pre-requisite for the identification of novel, potentially effective agents, based on thorough investigation of the molecular basis of these diseases.

In the future, a more specific approach may be tailored to specific mutations or groups of mutations associated with cardiomyopathies, by screening large panels of candidate molecules in assays based on induced pluripotent stem cells isolated from human fibroblasts [101]. As shown recently, the possibility of modulating the activity of sarcomere contractile proteins, such as beta-myosin, is beginning to surface, with huge potential implications for treatment of DCM, HCM and heart failure in general [102]. Finally, during heart development, immature cardiomyocytes proliferate actively to accommodate increasing heart size and function [103]; however, this proliferation is abruptly abrogated shortly after birth, leaving the heart with a limited regenerative capacity insufficient to replace substantial amounts of tissue lost after injury. For conditions characterized by loss of viable myocardial tissue and dysfunction, such as DCM and ARVC, an promising approach is constituted by the possibility of reactivating the dormant proliferative capacity of adult cardiomyocytes as a direct effect of miRNA delivery. In a recent study, selected miRNAs showed the ability of selectively induce cardiomyocyte proliferation in vitro and in vivo. miRNA delivery to the infarcted heart resulted in structural and functional recovery. Therefore, the broader action of miRNAs impacting multiple pathways opens up a new translational perspective for the treatment of complex cardiac disease as a stand-alone therapy or in combination with other regenerative resources. Likewise, control of gene transcription by specific interaction with histone acetylation and deacetylation (by the antagonistic families of histone acetyltransferases and histone deacetylases) may represent a viable therapeutic pathway in genetic heart disease, with targets ranging from modulation of hypertrophy to cardiac regeneration [104].

Overall, these broad concepts provide a broad intellectual framework supporting the idea of a common pathophysiologic "core" of primary heart muscle disease, representing a unitary target for research efforts in the field. Although the final application of future therapies will be necessarily individualized (i.e. both disease- and patient-specific), the beginning of this therapeutic revolution will be based on the comprehensive understanding of cardiomyopathies as a whole. Similar to what is happening in cancer or autoimmune diseases, it will be impossible to cure a single entity without being near to curing the rest. Rather than subdividing the field in water-proof niches with little communication, the approach to cardiomyopathies should constantly strive to pursue the inter-disciplinary cross-fertilization. It is hoped that the present work may contribute to this novel perspective.

1.4 Aim of the thesis and project design.

The present thesis reflects several aspects of my work at the Florence Referral Center for Cardiomyopathy and Careggi University Hospital during the last 3 years. The leading theme is the effort to embrace the complexities of cardiomyopathies using a translational approach ranging from clinical to imaging, to genetics, to basic science.

Section 2 focuses on genotype-phenotype correlations in two specific disease models (thin filament-associated HCM and Anderson Fabry disease) and the impact of next generation sequencing on the diagnosis of clinically challenging cardiomyopathies. Section 3 deals with the hypothesis that environmental modifiers may exert a significant impact on phenotype and clinical course, by addressing one of the most prevalent cardiovascular risk factor in the western world, i.e. obesity, in HCM patients undergoing cardiac magnetic resonance imaging. Section 4 addresses clinical markers of risk and predictors of outcome in patients with HCM (by evaluating the value of NT-pro BNP and late gadolinium enhancement as a clinical barometers of disease progression and arrhythmic risk), DCM (assessing the impact of advances in management on outcome), and an acquired model of myocardial disease involving cell-mediated immunity – Chagas cardiomyopathy. Section 5 illustrates novel therapeutic approaches that are being developed for patients with HCM and Anderson-Fabry disease, and a critical reappraisal of a well-established – but not perfect – preventive option such as the implantable cardioverter defibrillator. Finally, in my conclusive remarks, I will outline work that is presently ongoing and future directions for research to be pursued in Florence.

2. Genotype-Phenotype Correlations

"The important thing in science
is not so much to obtain new facts,
as to discover new ways
of thinking about them"

Sir William Bragg

Genetics is a new science. It is little more than a century since Mendel's laws were rediscovered in 1900, and less than 60 years since the structure of DNA was discovered in 1953. Human and medical genetics were late developers: they started to slowly develop during the first half of the 20th century, then saw an increasingly rapid rise and continues in the 21st century. Thanks to the outstanding efforts of *Victor Almon McKusick*, now regarded as the "father of medical genetics", in the second half of the 20th century genetics met medicine. Since then, the Holy Grail in medical genetic has been the ability to deduce the clinical phenotype of an individual from his genotype. It was once naively assumed that, at least for ''monogenic'' disorders, genotype–phenotype relationships would be simple and straightforward to understand. This era of substantial optimism conquered the whole word of medicine: physicians and geneticists shared the unrealistic expectation that molecular genetics would lead to a new paradigm in predicting the outcome of patients.

What was initially thought to be one-to-one gene-disease has turned out to display important variability, dependent in large part on the genetic and environmental backgrounds into which the genes express. Therefore, the reality is that we cannot readily draw straight lines of causation from known genotypes to specific clinical phenotypes [Fig 2-1]. Although the original goal to link genotype to phenotype remains, it is now clear that the overall complexity of this relationship will require a far more subtle understanding of both molecular mechanisms and clinical correlates. Furthermore, there is an additional and perhaps central issue that compromise our ability to match genotype to phenotype: the progressive nature of the cardiac pathogenic process. Longitudinal studies of patients with cardiomyopathies have documented the dynamic nature

of the ventricular remodeling. Thus, it is apparent that focusing only on the end phenotype as the supposed "link" to the molecular mechanism is not only limiting but also likely to be misleading.

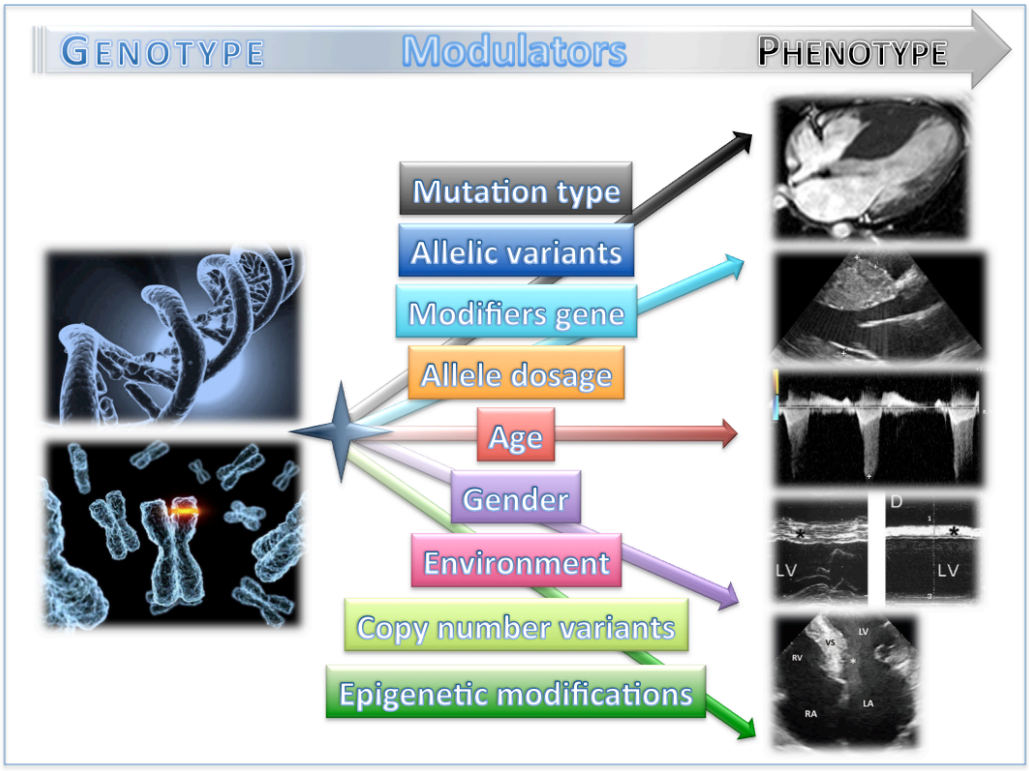


Figure 2-1: Genotype-phenotype correlations

The connections between phenotype and genotype are complex and quite hard to understand. Even in monogenic disorders, the same genetic variant can lead to different clinical pictures. The expression of phenotype may be modulated by a variety of genetic and non-genetic factors. Among the former, the type of mutation, the phenomenon of allele dosage, the presence of modifiers gene and epigenetics modifications are those better known. Environmental factors are those who are modifiable and can be have a vigorous influence on phenotypic expression (see chapter 3).

2.1 Into the myocardium: the distinct phenotypic expression of thin filament mutations in hypertrophic cardiomyopathy

It was in a cold and rainy day of November that we first have the idea of designing this study. At the end of a very busy morning in the outpatient clinic, we were reviewing the most interesting cases of the day. That morning we evaluated, totally by chance, three patients with HCM due to mutations in the thin filament genes. We were discussing the patients, their clinical history and disease progression. We were also analyzing echo images, one after another, and in that moment we realized how they were part of a distinct subgroup, if compared to other patients with mutations in thick filament genes.

HCM is a disease of the sarcomere: mutations are very often found in one of the two genes encoding for thick filament proteins (myosin binding protein C -MYPC3 and myosin heavy chain - MYH7) [Fig. 2.1-1] [1]. Mutations in the thin filament regulatory protein genes accounts for a minority of molecular defect involved in HCM and includes cardiac troponin T (TNNT2) and I (TNNI3), alpha-tropomyosin (TPM1), cardiac actin (ACTC) and, very rarely, troponin C [2]. These mutations, although rare, have always seduced physicians and geneticists involved in the field, because of them ominous outcome and high prevalence of malignant arrhythmias especially in the young.

Prior reports of patients with thin filament mutations described a severe form of HCM characterized by early onset, mild degrees of hypertrophy and high prevalence of juvenile sudden cardiac death [3-6]. Despite the low statistic power of these studies, limited by sample size

and a cross-sectional design, such rumor spread quickly among cardiologists, and these mutations are now known as “malignant mutations”. However, recent reports have shown that the overall spectrum of thin filament-HCM is far more heterogeneous than previously thought, extensively overlapping the more prevalent forms of HCM associated with thick filament mutations [7].

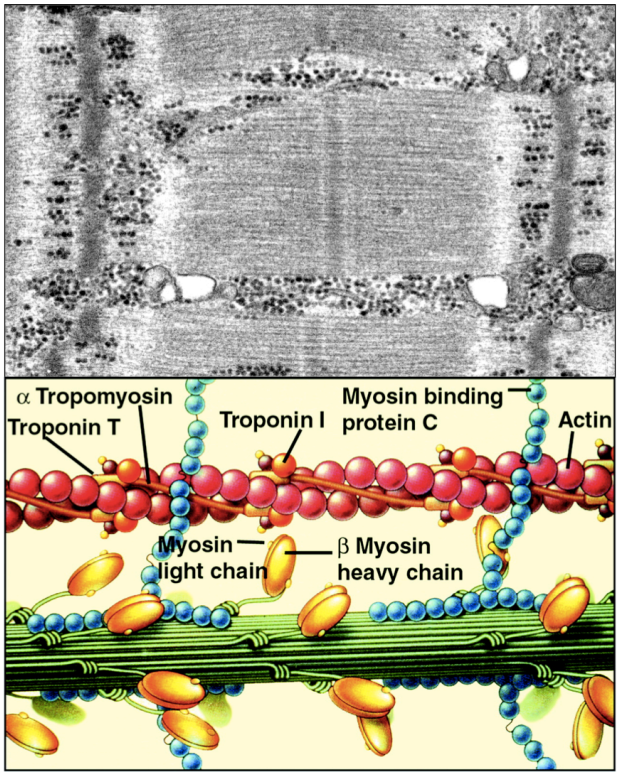


Figure 2.1-1: The sarcomere

The upper panel shows a transmission electron micrograph of a human cardiac sarcomere. The lower portion of the figure shows a simplified illustration of the sarcomere.

Two important questions arise from these considerations: is thin filament HCM a distinct disease? How and why is thin filament HCM different from the more common thick-filament disease?

Given the international network in which our center was involved, we decided to design a multicenter study, in order to describe the clinical features and outcome of a large cohort of patients with thin filament HCM, compared to matched patients with pathogenic thick filament mutations. The study cohort comprised 84 patients with a clinical diagnosis of HCM associated with one or more thin filament gene mutations (including cardiac troponin T (*TNNT2*) and I (*TNNI3*), tropomyosin (*TPM1*) and cardiac actin (*ACTC*), while no troponin C mutations were found), while 157 HCM patients with pathogenic mutations in the thick filament genes (*MYBPC3* and *MYH7)* were used as controls [Fig 2.1-1]. Four referral centers for cardiomyopathies were involved in the study: Careggi University Hospital in Florence, Italy; Brigham and Women's Hospital in Boston; Stanford Medical Center and University of Michigan Medical Center in Ann Arbor.

Patients underwent a thorough clinical and instrumental evaluation, including clinical history, echocardiography, 12-lead basal and 24-hours ambulatory ECG and cardiac magnetic resonance. They were followed-up at yearly intervals with review of history and symptoms, physical examination, and echocardiographic examination, and electrocardiography. 24- to 48-hour ambulatory ECG monitoring and CMR were performed if clinically indicated.

The phenotype of patients with thin-filament mutations slowly started to reveal itself. Compared with patients with thick filament disease, thin filament HCM seemed to represent a distinct and well-defined subset, in terms of cardiac morphology, systolic and diastolic myocardial function and patterns of disease progression.

2.1.1 Cardiac morphology and arrhythmic burden

The "thin filament" heart appeared to be less prone to develop severe hypertrophy. Mean maximal LV wall thickness values were on average milder than that observed in the thick filament group. Furthermore, the distribution of hypertrophy within the left ventricle was atypical, with high prevalence of concentric and apical patterns whereas thick filament HCM was almost uniformly (94%) manifest as classic asymmetric LVH involving the basal septum and anterior wall [Figure 2.1-2]. The atypical distribution of hypertrophy, together with the mild degree of hypertrophy, also accounted for the low prevalence of resting LV outflow tract obstruction.

The arrhythmic risk of HCM has been known since the first reports of the disease in the fifties. With time, due to the increasing knowledge of the complexity of this cardiomyopathy, many other features of the disease have been described and ventricular arrhythmias are now considered only part of a heterogeneous spectrum of characteristics, with an incidence of sudden cardiac death of 1%/year in non selected populations. The arrhythmogenic burden and the risk of sudden cardiac death of thin filament mutations have always gained most of the attention. Indeed, our results are consistent with previous reports of enhanced arrhythmic propensity associated with thin filament HCM. In our cohort, the annual rate of major arrhythmic events, including sudden cardiac death, resuscitated cardiac arrest and appropriate ICD intervention, was almost double that of the thick filament group (2%/year).

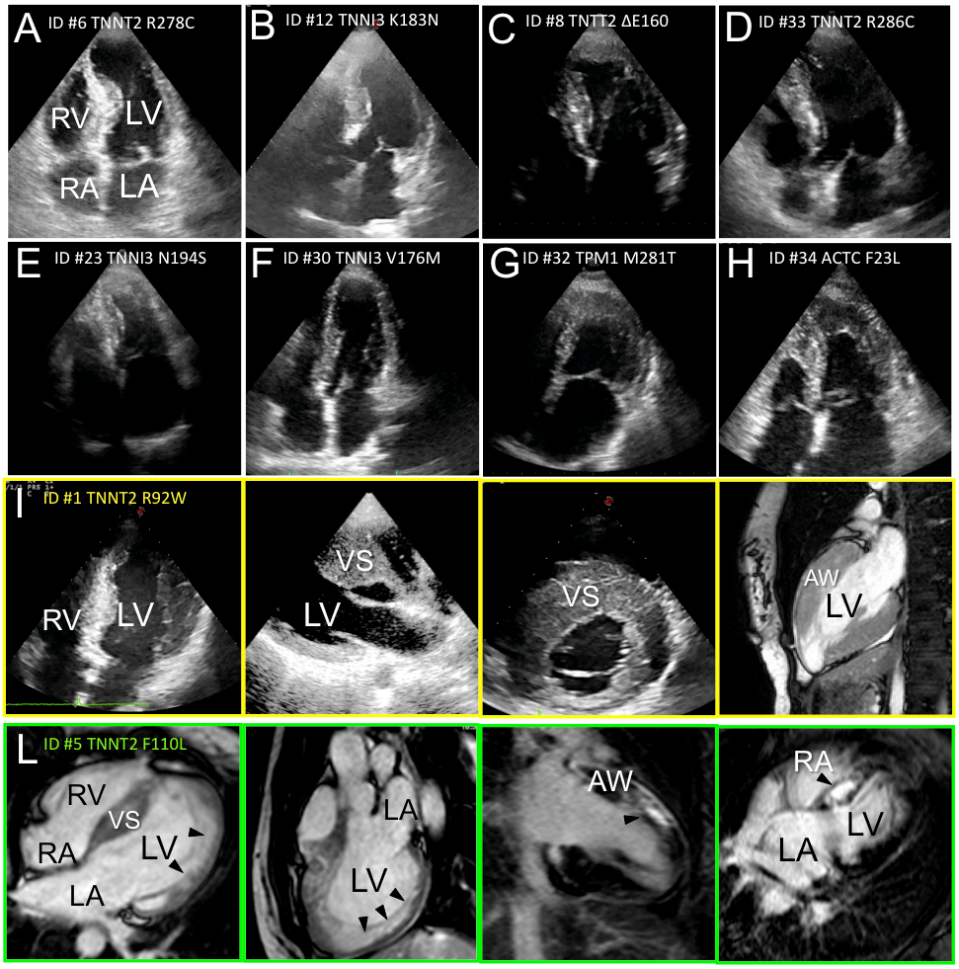


Figure 2.1-2: Phenotypic variability in thin filament HCM.

(A-H) Echocardiographic apical 4-chamber views showing different patterns of distribution of hypertrophy (ID number and mutation for each patient are reported).

(A-B) Mild-to-moderate hypertrophy, with "classical" septal localization of maximal LV thickness.

(C) Moderate septal hypertrophy with apical involvement;

(D) mid-apical septal hypertrophy;

(E) moderate mid-septal hypertrophy with severe left atrial dilatation;

(F) mild concentric hypertrophy

(G) apical hypertrophy with severe left atrial dilatation;

(H), moderate concentric hypertrophy with severe apical involvement and marked bi-atrial enlargement. (I) Echocardiographic and cardiac magnetic resonance (CMR) images from a patient with the TNNT2-R92W mutation. From left: apical four-chamber and parasternal long