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GENETIC SCREENING FOR MUTATIONS IN PATIENTS WITH INHERITED CARDIOMYOPATHIES USING NEXT-GENERATION SEQUENCING

by
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AN ABSTRACT OF THE THESIS OF

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Title: Genetic Screening for Mutations in Inherited Cardiomyopathies Using Next Generation Sequencing

Cardiomyopathy is a disease affecting the myocardium leading to its weakness and enlargement, consequently ending in heart failure or sudden cardiac death. Cardiomyopathy is classified to five types based on its manifestation and morphological features: hypertrophic, dilated, restrictive left ventricular, arrhythmogenic right ventricular, and left ventricular non-compaction. A genetic component has been associated with the disease and linked to its risk factors. Recently, mutations in one of a panel of culprit genes identified so far classified cardiomyopathy as a hereditary disease.

We have performed genetic screening for mutations in our populations, which have been only rarely studied, in a panel of the 34 genes implicated in cardiomyopathy phenotypes using Haloplex targeted next generation sequencing for 19 patients. We have used high throughput sequencing methods in which clonally amplified DNA templates of several cardiomyopathy patients were sequenced in a massively parallel fashion in a flow cell. Results have shown novel variants: *TGβ3* (C409R) (K407R) (K407Q) and (E169D), *DSP* (E1181D) *MYH7* (R1818L), *DES* (W295C), *VCL* (A259T), (R132L) and (R1055Q), *ABCC9* (R661C), (V822E) and (R824P), *RBM20* (R755C), *MYBPC3* (S217G) and (G1249C), *MYH6* (L772I) and (L1795M), *JUP* (T79P), *MYl2* (P95T), TCAP (A164E), and finally TNNC1 (G70D). Among our results there were non-synonymous nonsense mutations that resulted in stop codons and were of clinical significance. These are *TTN* (S14991*), *MYH6* (G1809*), and *LDB3* (C444*) and one frame-shift deletion in *MYH7* which resulted in alternative exon splicing. Seven patients, having idiopathic cardiomyopathy as the only cardiac disease, were whole-exome sequenced. We suspect to have three novel genes to be causing the cardiomyopathy disease: *LARP6* and *MYH15* both in a non-synonymous missense form and *AR* in a frame-shift form.

Genetic population studies will help in finding an effective and well-organized genetic counseling plan combined with a deeper and richer understanding of the associations and connections between the genotypes and phenotypes of the disease. Identifying a panel of genes associated with each type of the cardiomyopathy could be used as a promising genetic tool for early disease detection, prognosis, and management in the near future.

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ABBREVIATIONS

ACE	angiotensin converting enzyme
AF	atrial fibrillation
ARVC-ARVD	arrhythmogenic right ventricular cardiomyopathy
AV	atrioventricular
BSA	bovine serum albumin
CHD	coronary heart disease
CMR	cardiac magnetic resonance
DCM	dilated cardiomyopathy
ECD	enrichment control DNA
EMF	endomyocardial fibrosis
ESC	European Society of Cardiology
gDNA	genomic DNA
GS	gel-stain
HCM	hypertrophic cardiomyopathy
HT1	hybridization buffer
ICD	implantable cardio-defibrillator
ISFC	International Society and Federation of Cardiology
LVNC	left ventricular non-compaction cardiomyopathy
LVOT	left ventricular outflow tract
MDCM	mildly dilated congestive cardiomyopathy

NGS	next-generation sequencing
PPCM	peripartum cardiomyopathy
PVCs	premature ventricular contractions
RCM	restrictive cardiomyopathy
RE	restrictive enzyme
RPM	revolutions per minute
SD	sudden death
SCD	sudden cardiac death
SMVT	sustained monomorphic ventricular tachycardia
VF	ventricular fibrillation
VT	ventricular tachycardia
WHO	World Health Organization

CHAPTER I

INTRODUCTION

A. Cardiomyopathies

Back in 1980, the first report was issued by the World Health Organization (WHO)/ International Society and Federation of Cardiology (ISFC) task force defining cardiomyopathy as a disease of the heart muscle of unknown cause. It has classified this disease into three groups: dilated, hypertrophic, restrictive and a fourth group termed "unclassified cardiomyopathy" which covers the cases that do not fit readily into any group (Report of the WHO/ISFC, 1980). When this classification was proposed, there was lack of knowledge of the underlying causes leading to the different types of cardiomyopathies, yet scientists thought that those causes were distinct and unrelated. The disease was classified as primary when the cause was unknown, and secondary or specific heart muscle disorder when the leading cause was identified.

Later, after this disease has been more common and explored, WHO described cardiomyopathies as the group of diseases with strong genetic background that affect the myocardium and leads to cardiac malfunction (Richardson 1996). A committee from the American Heart Association proposed a new scheme of classification where primary cardiomyopathy was termed when the heart is the merely and primarily underlying cause of the disease, while secondary was given to the conditions where cardiac dysfunction is part of a systemic disorder (Maron 2006). This classification has faced difficulties due to the extrapolation of some diseases classified as primary to involve extra-cardiac organs;

contrariwise, diseases classified as secondary show at the pathology level to exclusively involve the heart.

Cardiomyopathies have a variety of causes, symptoms, and treatments. They are classified most recently into five groups according to the European Society of Cardiology (ESC) based on the morphological and functional phenotype: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restrictive left ventricular cardiomyopathy (RCM), arrhythmogenic right ventricular cardiomyopathy (ARVC), and left ventricular hypertrabeculated or non-compaction cardiomyopathy (LVNC) (Maron 2006, Charron 2010). Cardiomyopathies can occur at any age except for ARVC that occurs exclusively at young age (Towbin 2014).

The ESC classification has classified the cardiomyopathies into familial and non-familial forms neglecting the old nomenclature of primary and secondary cardiomyopathies. With the introduction of this new concept, they are orienting diagnostics towards genetic testing and emphasizing the role of genetics in this disease (Elliot 2008). In an attempt to direct diagnosis towards genetic studies, researchers have studied protein structure/function and disruptions that lead to an observed phenotype. They grouped genes into categories causing the same type of cardiomyopathies: genes causing arrhythmias are those coding for ion channels while disruptions in those coding for sarcomeric proteins are known to cause HCM. Mutations in sarcomere-sarcolemma link proteins are known to lead to DCM, while sarcomere signaling pathway proteins cause LNVC. Finally disturbances in desmosome proteins are the cause of ARVC (Figure 1).

High-throughput sequencing strategies are gaining lots of success in the clinical field especially in the cardiomyopathy disease since 9% of patients carry more than one

causative cardiomyopathy mutations (Kelly 2009, Zou 2013). The indexed patient along with first-degree relatives are screened through those technologies which are proved to be cost-effective (Ingles 2012). The benefits of genetic diagnostics in cardiomyopathies have reached the level where international guidelines are recommending effective gene diagnostics for these patients (Ackerman 2011). It is becoming an assertive diagnostic and prognostic tool in the field of cardiomyopathies.

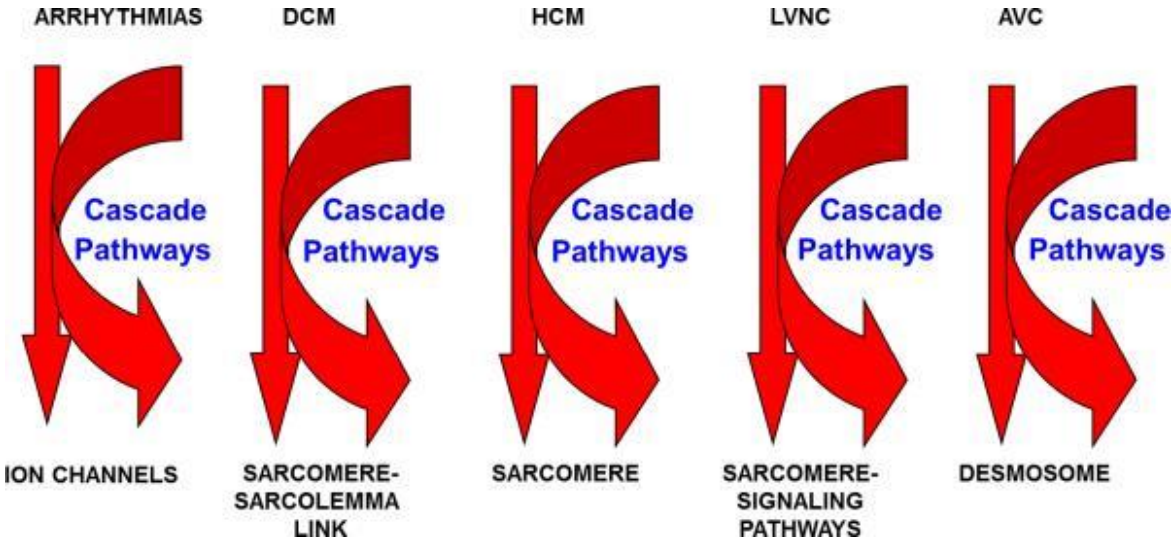


Figure 1. Final common pathway hypothesis
Source: Towbin, J. A. "Inherited Cardiomyopathies." *Circulation journal: official journal of the Japanese Circulation Society* 78.10 (2014): 2347-56.

B. Hypertrophic Cardiomyopathy

1. Epidemiology

Back in the 1950's the disease was first described by Teare who reported the death of young adults with no prior symptoms. He stated the presence of hypertrophied heart involving the basal and mid-part of the inter-ventricular septum and the left ventricular (LV) wall. All patients had similar pathologic picture, which defined the basic characteristic features of HCM (Teare 1958).

HCM is an autosomal dominant and a prevalent type of cardiomyopathy. The complexity of the disease lies in its genetic heterogeneity, broad spectrum phenotypic presentation, and the difficulty of finding a genotype-phenotype correlation. HCM affects 1 in 500 in the general population, 2 in 1000 of the young adults (<30 years of age) and has a 10-50 folds greater occurrence than other familial cardiovascular diseases (Maron 2004). The peril of this disease is in its strong association with sudden cardiac death (SCD) in young especially athletes (McKenna 1989, Maron 1993, Maron 2014). It has a higher incidence in men than in women (0.26:0.09%) and in blacks than whites (0.24:0.10%) (Maron 1995).

2. Symptoms and Diagnosis

Most patients with HCM remain asymptomatic and have a normal life expectancy. However, some patients might show symptoms of palpitations, exertional dyspnea, chest pain, systemic thromboembolism, and diminished consciousness. Those symptoms might be accompanied by intolerance to continuous exercise and heart failure symptoms (Maron 2006).

The most common phenotypic expression of HCM is asymmetric hypertrophy of the inter-ventricular septum, with or without LV outflow tract obstruction. The highest percentage of patients present with LV obstruction of around 70%, mild to moderate left atrium dilation, microvascular dysfunction and myocardial bridging (Keren 2008, Bogaert 2014). Although LV hypertrophy is a hallmark for diagnosis, the phenotype includes myocyte disarray, fibrosis, microvascular remodeling, abnormalities of papillary muscles and mitral apparatus and myocardial crypts (Thaman 2004, Harris 2006, Olivotto 2012, Bogaert 2014). Thus, the pathophysiology of HCM is varied and complicated and it manifests itself in diverse ways to include left ventricular outflow tract (LVOT) obstruction, diastolic dysfunction, myocardial ischemia and arrhythmias (Wigle 1995). LVOT obstruction might be triggered by day to day activities or strenuous exercise (Geske 2009).

Historically the diagnosis of HCM was made through the incorporation of examination with electrocardiogram, and invasive angiographic procedures (Braunwald 1964). Today, the diagnosis is traditionally made noninvasively by echocardiography and more conventionally with magnetic resonance (MR) imaging. The latter is the most accurate method to get a precise size measurement of the wall thickness of the LV region. Thus, the most common way of diagnosis is through an echocardiography (Figure 2) and more informatively through magnetic resonance imaging providing information on the cardiac phenotype, its functional and hemodynamic characterization, presence and extent of microvascular dysfunction, and myocardial fibrosis. HCM is diagnosed when wall thickness measures 15 mm or more. Borderline wall thickness (12-15 mm) is difficult to

diagnose and should be accompanied with family history and other factors to increase the likelihood of HCM diagnosis (Bogaert 2014). Combining exercise with echocardiography is an essential tool for revealing HCM especially in patients with no LV hypertrophy when at rest (Gersh 2011). Morphologically, HCM is diagnosed when other cardiac diseases are ruled out in the presence of a hypertrophied septum or lateral wall of the left ventricle (Bogaert 2014). Thus when the clinical profile or any family history is accompanied with wall thickening of the left ventricle, suspicion of HCM is high.

Furthermore, genetic testing has become a key tool for HCM diagnosis and after being commercially present, it is now considered a confirmative test and a useful test for the identification of affected relatives in families with known genetic mutations (Christiaans 2009).

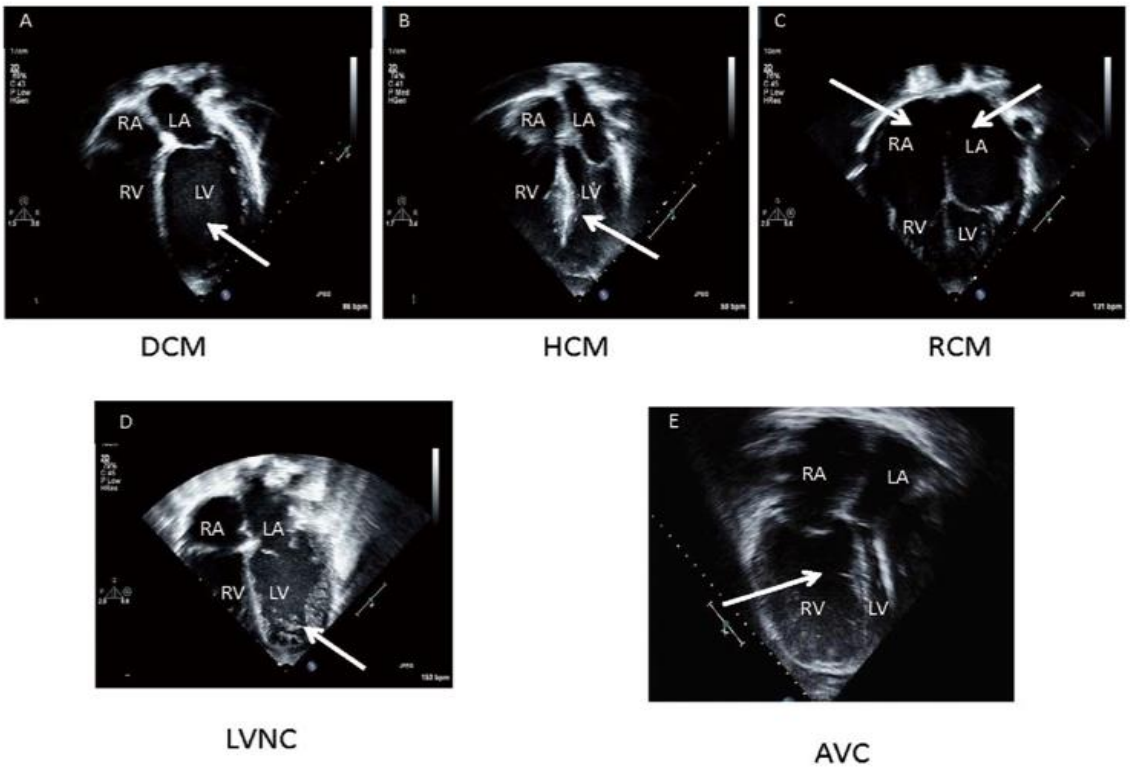


Figure 2. Echocardiographic features of cardiomyopathies. *Source*: Towbin, J. A. "Inherited Cardiomyopathies." *Circulation journal: official journal of the Japanese Circulation Society* 78.10 (2014): 2347-56.

3. *Genetics*

DNA methodology studies and molecular analysis back in early 1990 revealed that HCM is caused by a dominant missense mutation in the β-myosin heavy chain gene (*MYH7Arg 403 Gln*) on chromosome 14 (Jarcho 1989, Geisterfer 1990). Extensive research in the field to date revealed the involvement of 11 or more genes with >1400 mutations encoding sarcomere proteins, Z-disc or intracellular calcium modulators proteins responsible for the cause of HCM (Figure 3). Among these genes are the genes encoding

the β-myosin heavy chain, cardiac myosin binding protein C, troponin T, troponin I, alpha tropomyosin, actin, regulatory light chain, and essential light chain. The two most common genetic mutations, accounting for 70% of the mutations, are genes encoding for the β-myosin heavy chain (*MYH7*) and myosin-binding protein C (*MBPC3*), while the percentage of patients with the other gene mutations is less accounting for less than 1-5% (Bowles 2000, Maron 2012). The majority of mutations in the *MYH7* are missense, however, deletions and premature termination codons have also been identified. Mutations in the *MBPC3* are insertions, deletions, or frameshift mutations that result in truncation of the cMyBP-C protein with loss of function (Fatkin 2002). Some studies are investigating whether the harboring of more than one mutation can increase the severity of prognosis of the disease. Data compared between patients with homozygous, compound heterozygous, and double heterozygous compound mutations revealed that the clinical features of patients with more than one mutation led to increase in phenotype severity of the HCM manifested in greater risk of sudden death (SD) and LV hypertrophy (Kelly 2009). The consequences of mutations in the genes of the sarcomere result in proteins that activate myofilament drastically leading to myocyte hypercontractility and higher energy usage. These defects in the mitochondria of the myocytes lead to hypertrophic phenotypes. Furthermore, mutations in the intracellular calcium cycling proteins would alter the energetics of the myocyte resulting in decreased myocyte relaxation, myofibril disarray, and myocardial fibrosis (Watkins 2011). Mouse models of HCM show that the increased calcium concentration during diastole is likely to lead to signaling pathways that alter the physiological state leading to arrhythmias (Knollmann 2003, Huke 2010). A study has showed the association of a polymorphism in the 3' untranslated region of Angiotensin II type 2 receptor with LV

hypertrophy (Carstens 2011). Another study has revealed that resistin, a novel cytokine which was previously suspected to induce hypertrophy in rat cardiomyocytes, is increased with patients with HCM compared to controls (Hussain 2010). Moreover, polymorphism in calmodulin III gene was suspected to be a modifier gene in HCM (Friedrich 2009). Other gene mutations include ACTN2 which encodes alpha actin 2 (Chiu 2010) , *ANKRD1* which encodes cardiac ankyrin repeat protein (Arimura 2009), and *PRKGA2* which encodes the gamma subunit of AMP-activated protein kinase (Blair 2001). All this evidence shows that there are different mechanisms and pathways involved in HCM. These pathways potentially affect signals common to the downstream consequences of the myofilaments mutations (Watkins 2011).

Genetic screening, family history and pedigree analysis are essential to detect family members with no phenotypic expression of HCM. Identifying those carrying a mutation will facilitate their management and counseling. A list of genes is now used worldwide as a blueprint for all cases of cardiomyopathies including HCM (Figure 3).

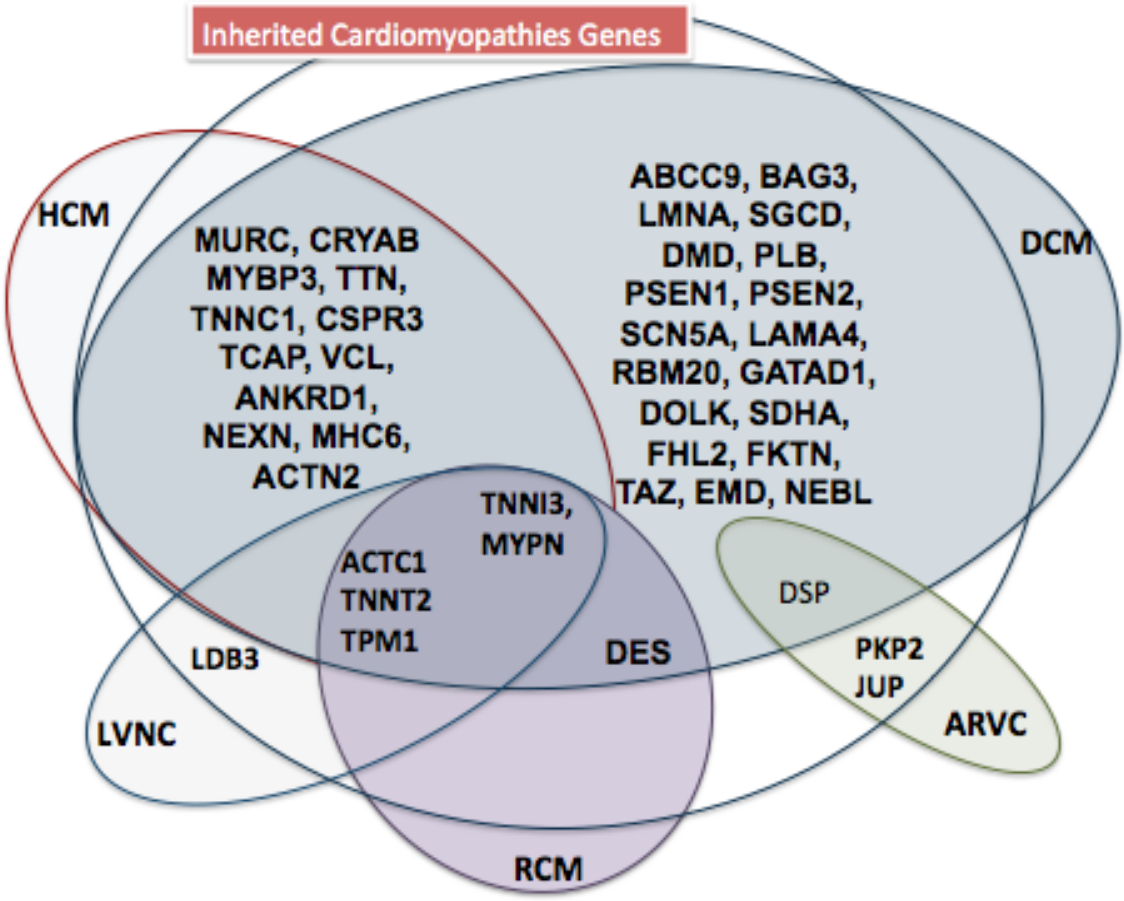


Figure 3: Summary of known genes implicated in cardiomyopathies

4. Complications

Since HCM is a heterogeneous hereditary disease with broad spectrum phenotype, its implications and manifestations differ widely in patients. Some patients progress to have serious complications mainly SCD (Moolman 1997, Spirito 2000 , Maron 2014), heart failure with exertional dyspnea and chest pain, and atrial fibrillation with embolic stroke (Maron 1999, Maron 2002).

One of the most unpredictable and serious complication of HCM is the SD accounting to 1-2% annual mortality rate in children and 0.5-1% in adults. Its risk lies in the fact that it could be the primary manifestation of the disease with mild or no prior symptoms (Maron 2013). SD mainly occurs in children and young adults, but it is not particularly restricted to an age group. Highest risk in SD of HCM patients has been associated with specific markers: magnitude of the hypertrophy has shown to be directly related to increased risk of SD (Spirito 2000), prior cardiac arrest or ventricular tachycardia, prior family history of HCM causative death, exertional syncope, and hypotensive blood pressure upon physical effort (Maron 2013). There has been a proposed correlation between the inherited genetic mutation of a patient and increased risk of SD. Patients with mutations in the beta myosin heavy chain and troponin T mutations have been documented to have a higher premature death than patients with other mutations (Geisterfer 1990, Watkins 1995, Niimura 1998).

Shortness of breath during exercise or exertional dyspnea, attacks of severe shortness of breath and coughing mainly occurring at night or paroxysmal nocturnal dyspnea, and extreme fatigue are another set of complications caused by HCM. These symptoms are signals of heart failure that can occur at any age of the affected patient. The main cause behind heart failure is the dynamic LV outflow obstruction or systolic dysfunction in the absence of obstruction. Other causes might be myocardial ischemia, outflow obstruction, and atrial fibrillation (Nihoyannopoulos 1992, Olivotto 2001).

A common arrhythmia and consequent complication seen in HCM is atrial fibrillation (AF). It is a condition of abnormal heart rhythm identified by pulse assessment or by an electrocardiogram where P waves are absent indicating AF. It has an occurrence

rate of 20-25% in HCM patients and is mainly accompanied by embolic stroke and accounting to 1% of annual death rate and disability (Maron 1999, Maron 2002). AF maybe the cause of heart failure especially when manifested before the age of 50, accompanied by basal outflow obstruction (Olivotto 2001). Studies on HCM correlation with AF revealed that patients with AF had worse symptoms, worse exercise capacity and a significantly higher risk of death from any cause compared to patients without AF, even after accounting for known risk factors of mortality in HCM or use of antithrombotic, antiarrhythmic, and septal reduction therapies (Siontis 2014).

5. Therapy and Prevention

HCM patients can be clinically classified into subgroups for treatment and management, yet those groups are not mutually exclusive and overlap between groups might occur. Patients who are genotype positive phenotype negative should always be followed up since they might undergo a conversion to phenotype positive with LV hypertrophy. Patients with none or mild symptoms should follow a drug therapy of beta blockers, calcium channel blockers, disopyramide, and/or diuretic agents. Patients with progressive heart failure symptoms should also be on beta blockers, disopyramide, and/or diuretic. Patients with none or mild symptoms might develop atrial fibrillation with time or become at high risk to SD (Maron 2013).

The decision for a cardioverter – defibrillator (ICD) implantation for primary prevention is based on the HCM-Risk SCD Score which depends on the maximal left ventricular wall thickness, left atrial diameter, maximal LV outflow pressure gradient, family history of SCD, non-sustained ventricular tachycardia, unexplained syncope, and

age (Elliot 2014). An ICD should be considered with 5-year SCD risk ≥ 6% and an ICD may be considered with 5-year SCD risk ≥ 4% and ≤ 6%. The HCM Risk-SCD should not be used in patients < 16 years, elite athletes, in individuals with metabolic/infiltrative diseases (e.g. Anderson-Fabry disease) and syndromes (e.g. Noonan syndrome), before and after myectomy or alcohol septal ablation. The HCM Risk-SCD should be used cautiously in patients with a maximum thickness ≥ 35 mm (Elliot 2014). An ICD is recommended for secondary prevention of HCM patients in the setting of cardiac arrest due to VT or VF and spontaneous sustained VT causing syncope or hemodynamic compromise and a life expectancy >1 year.

It is recommended that the patient be assessed for SD at presentation and monitored continuously every 1-2 years or upon clinical episodes (Muramatsu 2014). Those with sustained tachycardia or prior cardiac arrest should have an implantation of ICD. Studies show that ICD interventions appropriately terminated ventricular tachycardia/fibrillation is shown in 20% of patients (Maron 2007). Yet the dilemma in decision, especially with patients aging 17 ± 5 years, lies in the increased risk (40%) of ICD complications typically inappropriate shocks and device malfunction (Maron 2013).

Patients with heart failure with exertional symptoms are conventionally treated with beta adrenergic blockers with or without obstruction. Patients with outflow pressure gradient at rest, severe heart failure and obstruction are recommended to be on disopyramide with a beta blocker and not verapamil which should be avoided with those patients. Exercise echocardiography is the preferred method for provoking outflow-tract gradients in patients with hypertrophic cardiomyopathy. On the other hand, patients with severe symptoms of heart failure accompanied by systolic dysfunction should be on

diuretics, vasodilators, and digitalis. Whenever pharmacological treatment fails with those patients and life threatening condition comes at stake, surgical intervention should be assessed whenever outflow gradient is 50 mm Hg and more (Maron 2006, MS Maron 2006).

The third protuberant complication of HCM is AF and is mainly associated with age and left atrial enlargement. Paroxysmal or chronic AF can affect the quality of life with multiple hospital visits and lower quality of life (Sherrid 2005). Patients with episodes of AF are managed by anticoagulation along with electrical cardioversion. Recurrences of AF are managed by amiodarone (Olivotto 2001). New therapeutic strategies for patients with HCM with AF are emerging like radiofrequency catheter ablation. Successful sinus rhythm restorations is achieved with a decrease of AF recurrence in two thirds of the tested patients illustrating hope of a better management.

B. Dilated Cardiomyopathy

1. Epidemiology

Dilated cardiomyopathy (DCM) is a heart condition characterized by dilation of the left ventricle mainly or both ventricles and reduced left ventricular systolic function with normal ventricular wall thickening. This will consequently lead to left ventricular systolic dysfunction probably resulting in cardiac thrombus formation and a risk of systemic embolization. Those consequences put DCM at the upper threatening scale of cardiac diseases both in adults and children with high rates of morbidity, mortality and hospital admissions (Mestroni 1999). The involvement of both ventricles is neither necessary nor sufficient in providing a diagnosis. DCM can lead to heart failure and represents the highest

incident indication for heart transplantation (Sugrue, 1992). DCM is the most common form of cardiomyopathy (accounting for about 55%) (Maron 2006) and the most prevalent dysfunction of the cardiac muscle especially in the pediatric population with a higher incidence at the first year of age (Nugent 2003). The annual incidence is 2 to 8/100,000 with an estimated prevalence of 1/2,500 population (Maron 2006). Recent studies are showing idiopathic DCM high prevalence to be approximately ≥ 1 in 250 individuals (Hershberger 2013). Its predominance, similar to HCM, is higher in males than in females with a rate of 3:1 (Codd 1989, Rakar 1997). DCM can be encountered at any age, although most patients are diagnosed between 20-50 years of age (Dec 1994). The most common form is idiopathic followed by familial cases (Towbin 2006). In the Western population, 25% of patients have evidence for familial disease with mostly autosomal dominant method of inheritance (Burkett 2005). Inborn errors of metabolism cause familial forms in 6.8% of cases; these include metabolic disorders, oxidative phosphorylation defects and systemic carnitine deficiency (Cox 2007). Hormonal or nutritional conditions might lead to reversible dilated cardiomyopathy in the pediatric population; an example is vitamin D deficiency (Maiya 2008).

Mildly dilated congestive cardiomyopathy (MDCM) is a form of DCM where patients have advanced heart failure and severe left ventricular systolic dysfunction without significant left ventricular dilation. 50% of those patients have a family history of DCM (Keren 1990). Another encountered form of cardiomyopathy is peripartum cardiomyopathy (PPCM) presented by signs and symptoms of cardiac failure in the last month of pregnancy. PPCM can occur at any age not correlated to gravida with a higher prevalence in women

older than 30 years. It is more common in twin pregnancies, gestational hypertension, and anti-contraction medications and labor repressants (tocolytics) (Elkayam 2005).

2. Symptoms and Diagnosis

75 to 85% of patients present with heart failure as the primary most common symptom. Left-sided heart failure predominate, with diminished exercise capacity, progressive exertional dyspnea, and eventually orthopnea and paroxysmal nocturnal dyspnea. Dyspnea on exertion is seen in 86% of patients, 30% presents with palpitations, 29% with peripheral edema and 4 to 13% remain asymptomatic (Diaz 1987, Komajda 1990).

The key diagnostic feature in DCM patients is the left ventricle involvement with or without a dilated and dysfunctional right ventricle (Figure 2). Yet diagnosis is not made without referring to etiological assessment. DCM is generally subdivided as being either idiopathic or familial (Yancy 2013). Idiopathic DCM is allotted to a case when no identifiable cause is present after etiological assessment along with the identification of systolic dysfunction and a morphologically enlarged left ventricle. Thus, the criteria for the diagnosis of DCM are the presence of left ventricular fractional shortening <25% and/or LV ejection fraction < 45%, and LV end-diastolic dimensions > 117% of the normal value (Mestroni 1999). The idiopathic category is the trigger for genetic studies and family screening to possibly reach a familial DCM diagnosis using screening methods for first-degree relatives that include echocardiography and genetic tests (Hershberger 2013). Familial DCM by consensus now refers to patients with a genetic background yet

classification is based on pedigree analysis and phenotype and not solely on genetic tests. The presence of two or more affected individuals in a family, SD of a first degree relative at an age of 35 or less, history of cardiac transplantation, neuromuscular disease, or implantation of a pacemaker or cardio-defibrillator suggest a familial DCM (Hershberger 2010). Systemic arterial hypertension, coronary artery disease, valvular diseases, active myocarditis, chronic excess of alcohol consumption, sustained and rapid supraventricular arrhythmias, systemic disease, pericardial diseases, corpulmonale, congenital heart diseases are all potential causes for ventricular dilation, thus they should be excluded before the diagnosis of DCM. The idiopathic and familial forms of DCM are so similar in clinical presentation and management, the only difference lies in the earlier age of onset and higher left ventricular ejection fraction in the familial form (Mestroni 1999).

3. Genetics

A huge genetic heterogeneity illustrates DCM and accounts for half of the cases whereby 40 causative genes have been identified to date (Herhsberger 2013). These genes encode proteins of the sarcomere (the force that generates the structure of a cardiomyocyte), Z-disk proteins (cytoskeleton), nuclear envelope, heat shock chaperones and sarcolemma (cardiomyocyte membrane); consequently, mutations in them alter the cellular structures and muscle contraction (Figure 4). Other proteins encoded by those genes are ion channels and intercellular junction proteins that affect the functioning of ion channels, calcium homeostasis and sensitivity and force generation in the myocardium (Mestroni 2014). The pattern of inheritance in the genetic forms of DCM differs between age populations. The main pattern of inheritance in pediatric patients is autosomal

recessive, while in the adult population, familial genetic forms of DCM account for 30–48% of cases with autosomal dominant inheritance pattern (56%) (Mestroni 1999).

Several gene mutations are involved in DCM with a huge heterogeneity in the place of the mutation on the gene (Figure 3). Thus, sequencing of the entire coding region of the gene is a hallmark to identify disease-causing mutations (Hershberger 2013). Similar to HCM, mutations in genes coding the sarcomeric proteins cause DCM but less prevalently. The difference lies in the fact that DCM sarcomeric mutations reduce sarcomeric contractile function while HCM sarcomeric mutations amplify force contraction through gain of function (Lakdawala 2012). Thus the same mutation can yield different phenotypes and can manifest itself as HCM in one patient and DCM in the other. Examples of those genes are those encoding myosin, actin, tropomyosin, and titin proteins (*MYH6, MYH7, MYBPC3, ACTC1, ACTC2, TPM1, and TTN*). TTN mutations, which encodes the giant protein titin, are major causes of DCM (Norton 2003) . In a cohort of 312 individuals with DCM, nonsense, short insertion or deletion, or splice-site variants in *TTN* were associated with 25% of familial and 18% of sporadic DCM (Herman 2012). Mutations in the genes that encode the phospholamban (*PLN*), which is an important protein in the regulation of intracellular calcium, are known to cause DCM (Chen 2005). Desmosomal junctions proteins, which assist in force transmission during muscle contraction, including desmocollin-2 (*DSC2*), desmoglein-2 (*DSG2*), desmoplakin (*DSP*), junction plakoglobin (*JUP*), and plakophilin-2 (*PKP2*), are known to cause ARVD and DCM (Garcia-Pavia 2011). Other gene mutations associated with DCM are *LMNA* gene coding nuclear proteins lamins A and C which form the lamina of the nuclear envelope (Sylvius 2010). DCM phenotype caused by *LMNA* mutation is seen in almost all patients with conduction system

disease, atrial or ventricular arrhythmias, or ventricular dysfunction (Brodt 2013). Mutations in *LAMA4*, an extracellular-matrix protein, have been found in patients with DCM as well (Wang 2006).

DCM causing mutations are, moreover, coupled to ion channel genes like sodium channels genes (*SCN5A)* (McNair 2004), cytoskeletal proteins coding genes like desmin (*DES)* which is the chief intermediate filament of skeletal and cardiac muscle. It plays an important role in maintaining the structural and functional integrity of the myofibrils. Mutations in DES can cause a wild range of phenotypes one of which is DCM (Taylor 2007). Another cytoskeletal protein coding gene mutation linked to DCM is Cypher/ZASP (*LDB3*) which codes for a protein having a PDZ-interacting domain. *LDB3* mutation is also associated with another type of cardiomyopathy, which is the non-compaction type (Vatta 2006). Recent studies are linking DCM to new genes like *BAG3* encoding BAG family molecular chaperone regulator 3. This protein has anti-apoptotic activity and localizes at the Z discs of striated muscles. Researchers are suggesting that the mutation is interfering with Z discs assembly and causing apoptosis of cardiomyocytes leading to DCM (Arimura 2001). Another gene linked to DCM through extensive research is the gene coding the RNA binding motif protein 20 (*RBM20) w*hich is phosphoprotein that is primarily expressed in the heart muscle (Li 2010). Mutations in RBM20 were observed in approximately 3% of subjects with DCM (Refaat 2012).

Most DCM-associated mutations are nonsynonymous missense that is they produce a protein with a different amino acid from that in the native version. Yet, nonsense, frameshift, insertion or deletion, and splice-site variants have been documented. Most DCM associated mutations are specific and unique to the family and often reported for the first

time. Heavy genetic research work is being conducted in the field linking so many genes to DCM. Only 40% of cases so far are linked to genetic causes and all the rest are considered idiopathic (Hershberger 2013).

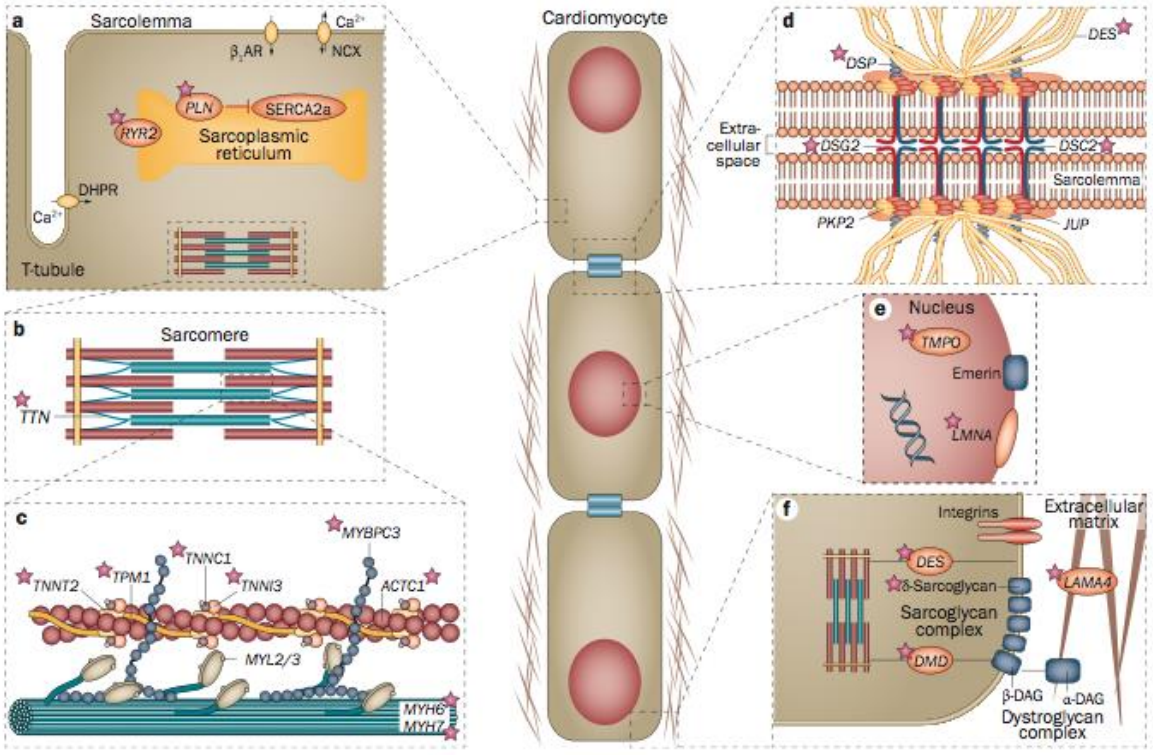


Figure 4: Genetic diversity in dilated cardiomyopathy
Source: Hershberger R.E., D.J. Hedges, A. Morales. "Dilated cardiomyopathy: the complexity of a diverse genetic architecture." *Nat Rev Cardiol* 10 (2013):531–547

4. Complications

The clinical course in adults and children patients of DCM is mostly unpredictable. Serious atrial arrhythmias presented early in the disease are reported with patients of DCM mainly with inflammatory processes (giant cell myocarditis and sarcoidosis) or with *LMNA* mutations (Kumar 2015). DCM is featured by left ventricle dilation as mentioned earlier, thus this imposes a serious conduction system problem. Patients with DCM have an intra-ventricular delay in the left ventricle or dyssynchrony resulting in reduced ventricular systolic function and altered myocardial metabolism. Clinical manifestations in DCM patients are diverse and can advance to sinus node dysfunction, various degrees of atrio-ventricular (AV) block, inter-ventricular conduction delay, and bundle branch block. Disturbances in His-Purkinje conduction due to DCM can lead to SCD (Rapezzi 2013). Ventricular pre-excitation has been described in association with mutations in the *PRKAG2* gene that also cause conduction defects, increased ventricular wall thickness, and progressive left ventricular dysfunction (Gollob 2001).

Another set of complications are supraventricular tachyarrhythmia which may occur before ventricular dilation and they are caused by the development of atrial ectopy (an extra heartbeat caused by a signal to the upper chambers of the heart (the atria) from an abnormal electrical focus), atrial tachychardia (a type of atrial arrhythmia in which the heart's electrical impulse comes from ectopic atrial pacemaker rather than from the SA node which is the normal site of origin of the heart's electrical activity), and atrial flutter (an abnormal rhythm in the atria) (Lakdawala 2010). The common form of atrial flutter seen in DCM patients is right atrial flutter where an electrocardiogram shows classic saw-tooth flutter waves. This manifestation on an ECG suggests highly progression to AF, which is

consequently identified in most of these patients (Waldo 2008). AF usually develops in 30-40% of patients with congestive heart failure regardless of the direct etiology behind the cardiac failure (Stevenson 1999). Thromboemboli and stroke are seen in AF patients raising the rates of morbidity and mortality.

Patients with DCM can develop also ventricular arrhythmias including premature ventricular contractions (PVCs), sustained monomorphic ventricular tachycardia (SMVT), polymorphic ventricular tachycardia (VT), and ventricular fibrillation (VF) (Kumar 2015). Polymorphic VT and VF leads to cardiac arrest due to asystolic arrest and pulseless electric activity (Sen-showdhery 2012).

5. Therapy and Prevention

The American Heart Association has divided DCM patients into four groups based on their heart failure; stage A: patients at risk without symptoms or structural heart disease; stage B: presence of structural heart disease without symptoms; stage C: structural heart disease with present or past heart failure symptoms; and stage D: refractory heart failure (Jessup 2009). Angiotensin converting enzyme (ACE) inhibitors are given to stages B and C, along with β-blockers given to stage C patients to effectively reduce morbidity and mortality (Hunt 2009). Patients with a genetic background are put on the use of ACE inhibitors, angiotensin receptor blockers, β-adrenoreceptor blockers, aldosterone antagonists, and vasodilators. In addition, all family members that are identified as carriers or first-degree relatives of affected patient should receive genetic counseling to properly manage the disease and its onset (Mestroni 2014). Therapy with the vasodilator improved survival in patients with DCM when using combination of hydralazine and isosorbide

dinitrate, yet did not improve outcome when administering doxazosin and dihydropyridine calcium channel blockers (Thackray 2000).

Treatment of AF which is a serious complication of DCM is controversially through antiarrhythmic drug therapy like amiodarone. Some showed that the inability to maintain sinus rhythm by usage of amiodarone was ineffective and increased mortality by 49% (Wyse 2002), others concluded that when amiodarone was the major antiarrhythmic drug, the maintenance of sinus rhythm did not have any effcte (Talajic 2010). DCM patients who are at risk of AF and with left ventricular systolic dysfunction showed that upon treatment by catheter ablation had benefit on ventricular function, especially when performed early rather than later after development of AF and heart failure (Anselmino 2014). Amiodarone remains the major drug of choice when ventricular function is repressed. On the other hand, Sotalol and dofetilide might be drugs of choice for patients with acceptable ventricular and renal function and preserved renal function; however, those patients require follow-up for toxicities and potential proarrhythmia. DCM patients with AF should achieve an acceptable rate control (beats per minute). β-Adrenergic blockers are used as the first-line therapy to achieve this goal (Kumar 2015). As for preventing SCD, the ICD remains the most effective form of therapy in its prevention in patients with DCM. Survivors of SCD have a life expectancy of 12 months with an implantable ICD (Russo 2013). Finally, as the case with other cardiac diseases, excess dietary sodium, alcohom ingestion and exposure to cardio-toxins should be limited and avoided (Mestroni 2014).

C. Restrictive Cardiomyopathy

1. Epidemiology

Restrictive cardiomyopathy (RCM) is a type of cardiomyopathy where either one or both ventricles become stiff due to the replacement of the normal heart tissue with abnormal tissue. This will cause impaired ventricular filling and relaxation where the ventricles become filled with blood leading to atria enlargement, decreased blood flow in the heart, reduced diastolic function, and several complications. RCM is characterized by hemodynamic abnormalities, myocardial relaxation abnormality with interstitial fibrosis and calcifications. RCM manifests itself with a clinical heart failure syndrome frequently mistaken for that caused by systolic dysfunction, along with AV block and symptomatic bradycardia in some cases (Sasikan 2014). RCM is classified as primary or secondary to an underlying cause. It may result from radiation treatments, infections, or scarring after surgery. It has been classified as familial, idiopathic or can be the result from various systemic disorders, like amyloidosis, sarcoidosis, carcinoid heart disease, scleroderma and anthracycline toxicity (Elliot 2007). Clinically it is hard to be defined since restrictive ventricles occur in a wide range of pathologies (Kushwaha 1997). A consensus panel organized by the American Heart Association placed RCM in a mixed category of genetic or acquired disease. It was primarily thought to have a non-genetic cause yet as more research is conducted in the field, RCM is being categorized as a familial disease (Maron 2006).

RCM is the rarest type of primary heart muscle disease accounting for approximately 2.5-5% of all cases. It affects predominantly elderly but can occur at any age group. It is more prevalent in tropical Africa than in the Western world. Median survival

without transplantation is reported at around 2 years (Russo 2005). The estimated annual incidence in the United States is 0.04/100,000 children and that in Australia is 0.03/100,000 (Denfield 2010). The most common cause of RCM is endo-myocardial fibrosis (EMF). Affecting mostly children and adolescent, EMF affects 10 million people worldwide. The high incidence in some ethnic groups suggests a genetic background for the disease (Mocumbi 2008).

2. Symptoms and Diagnosis

Patients with RCM might present with variant symptoms including respiratory problems with recurrent lower respiratory tract infections, dyspnea upon movement and exercise reaching exercise intolerance (Denfield 2002). Additional signs are abnormal heart sounds, hepatomegaly, chest pain, syncope, and more serious complications like SD (Rivenes 2000).

The electrocardiogram of RCM patients reveals right or left atrial enlargement along with ST segment depression and ST-T wave abnormalities. In some patients also right or left ventricular hypertrophy and conduction abnormalities are seen (Denfield 2002). Arrhythmias have been reported in approximately 15% of pediatric patients and include atrial flutter, atrial fibrillation, atrial tachycardias, and symptomatic sinus bradycardia (Rivenes 2000). Echocardiogram is used to diagnose RCM where it shows prominently dilated atria in the absence of significant atrio-ventricular valve repetition along with restrictive filling and increased left ventricular end diastolic pressure (Figure2). In addition, cardiac catheterization is an important diagnostic tool showing pulmonary hypertension with elevated left or right ventricular end diastolic pressures (Cetta 1995). An accurate

diagnosis of RCM is crucial to distinguish this condition from constrictive pericarditis which is a condition that presents with restrictive physiology but is frequently curable by surgical intervention. This distinction is highly important for the treatment options and prognoses which differ drastically (Goldstein 1998).

3. Genetics

Several gene mutations causing other types of cardiomyopathies might also cause RCM (Figure 3). Desmin (*DES*), which was mentioned previously to cause DCM, might lead to RCM. Most *DES* mutations seem to be inherited in an autosomal dominant fashion and are associated with skeletal myopathy and cardiomyopathy (Kaski 2008). Myopalladin (*MYPN*) and β-myosin heavy chain (*MYH7 or MHC*) which both encode sarcomeric proteins, troponin I type 3 (*TNNI3*) mutations are thought to be associated with RCM (Towbin 2014). Some studies reported mutations in cardiac troponin I in RCM patients and implicated a poor prognosis (Mogensen 2003). Another study reported a positive cardiomyopathy family history where phenotypes varied between dilated, restrictive and left ventricular non-compaction. Mutations in this family were seen in troponin I (*TNNI3*), troponin T (*TNNT2*), and alpha cardiac actin (*ACTC*) (Kaski 2008). Z-disk protein-encoding genes, including *MYPN*, *TTN*, and *Bag3* have also been identified (Sen-Showdry 2010). Research in the field has shown that several gene mutations can lead to RCM and that there is a huge overlap between this type of cardiomyopathy in particular and the other types.

4. Complications

RCM patients have a very poor prognosis especially those presenting with syncope and chest pain. Those patients are at risk of SCD and heart related deaths (Rivenes 2000). Most patients have serious complications and receive transplantation within 3 years of diagnosis (Russo 2005). Cardiovascular mortality is high in RCM patients with congestive heart failure accounting for the majority of deaths. Children less than 5 years have several features of heart failure like cardiomegaly, pulmonary venous congestion on chest radiograph, and thromboembolism (Weller 2002). Atrial fibrillation was again a complication seen in 74% of patients along with systolic dysfunction in 16% of patients in a cohort study conducted. Endomyocardial histology was available commonly demonstrating interstitial fibrosis (81%), myocyte hypertrophy (86%), and endocardial fibrosis (45%).

5. Therapy and Prevention

There is no treatment or cure for RCM, yet patients are managed based on the symptoms they present with. Patients with respiratory problems are given diuretics. Those with thromboembolic events are give anticoagulants to manage them. RCM patients at an advanced stage with systolic dysfunction are put on ACE inhibitors. β-blockers are administered to patients with ischemia (Denfield 2010). Heart transplantation remains the most promising treatment since it results in longer survival (Weller 2002). Genetic mutation studies remain a promising field to identify patients at risk and potentially manage them with new targeted therapies.

D. Arrhythmogenic Right Ventricular Cardiomyopathy

1. Epidemiology

Arrhythmogenic right ventricular cardiomyopathy or dysplasia (ARVC/D) is a type of cardiomyopathy resulting from an abnormality in intercalated discs and desmosomes linking heart cells. Mainly the problem lies in the right ventricle with a possibility of the left ventricle involvement throughout the course of the disease (Frank 2013). ARVC defined histologically is the replacement of the right ventricular myocardium with adipose and fibrous tissue mainly in three sites: the right ventricular inflow, outflow, and apex (Elliot 2007) (Figure 5). The prevalence of ARVC varies within regions but it is 1:5000 in most cases (Saffitz 2011). 2-5% of SCDs in adults are linked to ARVC with high incidences in exertional deaths (Fraces 2006). Generally it is more prevalent in men than women (2-3:1). It mainly emerges during childhood and young adults (<35 years old), yet may also be manifested in elderly (Azaouagh 2011).

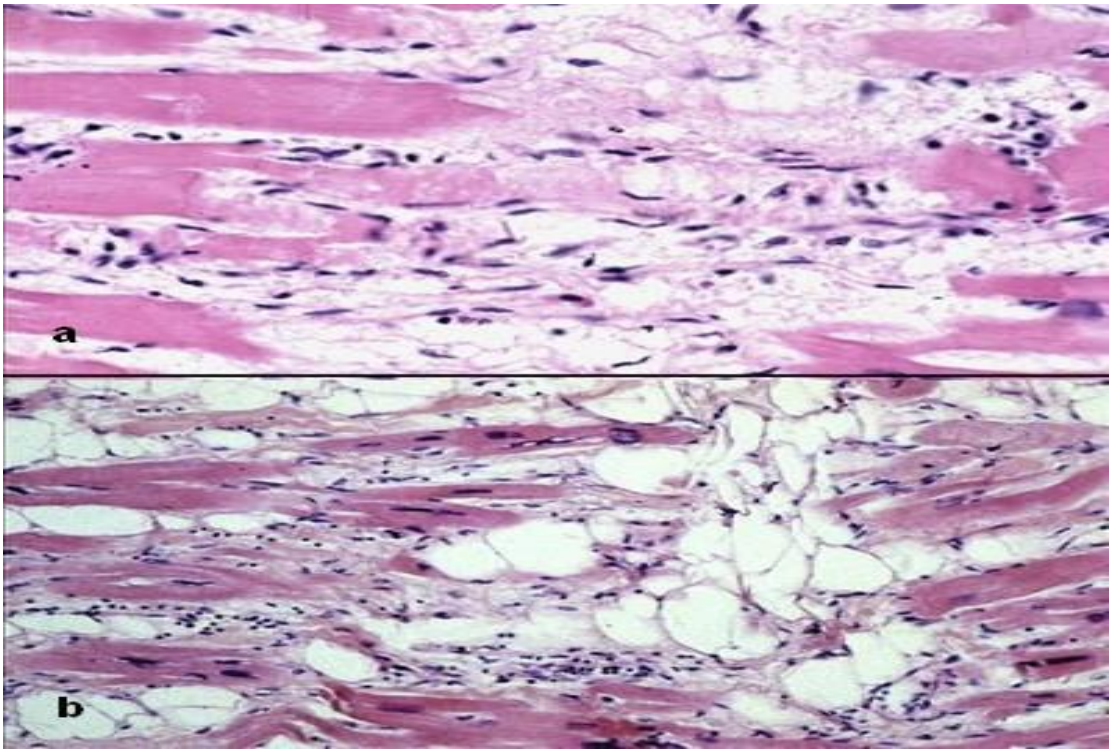


Figure 5: Histological features of Arrhythmogenic Right Ventricular Cardiomyopathy
Source: Thiene et al. "Arrhythmogenic Right Ventricular Cardiomyopathy/ Dysplasia**"**
Orphanet Journal of Rare Diseases 2 (2007): 45

2. Symptoms and Diagnosis

Clinical manifestations of affected individuals include electroanatomical abnormalities, ventricular arrhythmias and heart failure symptoms. Thus the initial symptoms are arrhythmias and conduction disturbances (Richardson 1996). Generally the condition is symptomless presented as SCD (Fraces 2006). The diagnosis of ARVC is made based on several parameters anatomical, histological, electrophysiological, arrhythmic and genetic features (Marcus 2010). Upon the incidence of SCD, the autopsy of the heart will show normal fat deposits in the right ventricle, and sub-epicardial scars of ischemic origin.

Histologically, the myocytes are destroyed *via* inflammation, scaring and fat replacement (Elliot 2007). Recently, cardiac magnetic resonance (CMR) is a noninvasive imaging tool able to combine both morpho-functional evaluation and tissue characterization, thus being useful for diagnosing ARVC (Sen-Chowdry 2007).

3. Genetics

ARVC has an autosomal dominant mode of inheritance with only 30-50% of patients being identified with gene mutations to be the causative agent of the disease (Fressart 2010). Thus, the research field with ARVC patients is extensively active for the possibility of finding new abnormal genes associated with the disease (Frank 2013). Desmosomal proteins are known to be mutated in ARVC; these include transmembrane desmosomal cadherins desmoglein 2 (*DSG-2*), desmocollin 2 (*DSC-2*), desmoplakin (*DSP*); and linker proteins such as junction plakoglobin (*JUP*) and plakophilin 2 (*PKP2*). The most reported mutation to cause ARVC is *PKP2* (Gerull 2004). It has been estimated that 60% of cases are linked to mutations in genes mentioned previously (Ackerman 2011). Yet, mutations in genes encoding proteins that interact with desmosomal proteins were found also to cause ARVC. These include desmin (*DES*) and titin (*TTN*) (Azaouagh 2011).

However, signs and symptoms of ARVC might not appear in patients with this genetic abnormality and it may require a second mutation in that specific gene or any related desmosmal gene for the patient to develop symptoms. Some mutated genes may increase the risk of developing ARVC yet may not be the causative genes by themselves, and thus the area of genetic studies in this disease is very active and interpretations are

critical. The clinical application of genetic studies lie in their aspect to help understand the cause of this disease and to identify family members who might be in danger to develop its symptoms (Frank 2013).

4. Complications

As mentioned earlier, SCD or aborted SCD is the primary symptom of ARVC in a high percentage of patients (23%). ARVC is a progressive disease which may advance to refractory VT or VF. The most common type of arrhythmia encountered in those patients is sustained or non-sustained monomorphic VT that originates in the right ventricle (Dalal 2005). Patients who have survived SCD are at 8-10% per year risk of recurrence and they are candidates for ICD (Sen-Chowdry 2007). The latter can implicate serious complications like myocardial injury or aneurysmal rupture. In addition, patients with ARVC requiring ICD are usually young, thus problems might arise in device implantation, pulse generator and functionality, in addition to psychosocial effects and consequences on quality of life (Romero 2013).

5. Therapy and Prevention

Sine ARVC patients present with a broad spectrum of symptoms ranging from asymptomatic to escaping SCD, treatment is individualized based on clinical presentation. Exercise has shown to worsen the arrhythmias seen in ARVC and lead to serious complications, thus patients are advised to avoid rigorous physical activities and sports (Maron 2005). Symptomatic patients with severe LV dysfunction and severe symptoms of

ventricular arrhythmias are treated with anti-arrhythmic drugs. β-blockers in combination with amiodaron and sotalol are effective treatments of sustained VT or VF. Patients with repetitive episodes of sustained VT or VF, unexplained syncope, family history of SCD are candidates for ICD. The latter is considered an effective treatment and preventive mechanism for SCD in ARVC patients (Maron 2006).

E. Noncompaction or Hypertrabeculated Cardiomyopathy

1. Epidemiology

Left ventricular noncompaction cardiomyopathy (LVNC) is an abnormality in the left ventricle where the compaction is incomplete causing trabeculations in the left ventricular mycoradium, and from here comes the second nomenclature of the disease: hypertrabeculated cardiomyopathy. The myocardial wall is thickened with a thin epicardial layer and a thickened endocardial layer (Hershberger 2009). A problem in the embryogenesis of the endocardium and myocardium is thought to be the cause of the disease. It is rare congenital cardiomyopathy affecting any age. The noncompaction mainly affects the left ventricle, but cases have been reported of the right or biventricular involvement. The prevalence of LVNC is unknown, yet based on echocardiograms the incidence is reported to be 0.05% of all adults' echocardiographic examinations (Towbin 2010).

2. Symptoms and Diagnosis

The clinical manifestation of LVNC is commonly similar to those encountered in congestive heart failure. On an echocardiograph, systolic dysfunction with dilated hypertrophic left ventricle is noted with deep trabeculations in the left ventricle apex and lateral wall (Ichida1999) (Figure 2). Features are not definite for the disease where patients may remain asymptomatic for the disease (Lin 2014). There are two types of LVNC: one that is presented as an isolated entity of any structural heart disease and is caused by an arrest of myocardial morphogenesis, and another type that occurs in conjunction with coronary heart disease (CHD) (Ichida 1999). Less information is available for this non-isolated form, yet the clinical presentation is similar to that of the primary myocardial form. There is no specific phenotype for LVNC patients, yet a wild range of phenotypes and presentations exist; the isolated LVNC has a normal left ventricle size and function with no arrhythmias. This group comprises 25% of all patients. The second type is the isolated form with arrhythmias in which there is a normal ventricle on an echocardiogram yet arrhythmias are common. The third type is the dilated form of LVNC that is so similar to the DCM. The differentiating feature is the ability of the heart to revert to a hypertrophic form or to a normal left ventricle size and function. The fourth type is the hypertrophic and dilated form of LVNC that is recognized clinically as the worst form and is associated with neuromuscular disease and hypotonia. The fifth type is the restrictive type of LVNC that is a very rare type and it mimics the clinical presentation of RCM. Finally the sixth type is the LVNC with CHD where any form of the latter can occur concurrently with LVNC (Towbin 2010). Echocardiography and cardiac magnet8ic imaging are again diagnostic tools for LVNC where non-compactions and trabeculations are seen in in the left ventricle apex and

lateral wall. The echocardiogram is abnormal and commonly has giant midprecordial voltages in around 30% of patients, specifically children (Pignatelli 2003).

3. Genetics

The American Heart Association classified LNVC as a primary genetic cardiomyopathy. 18-42% of cases are hereditary based on recent studies (Bhatia 2011). The disorder is inherited commonly as X-linked recessive or dominant. LVNC cases associated with CHD have a dominant mode of inheritance. 44% of LVNC patients have inherited LVNC, with 70% having autosomal dominant and 30% X-linked inheritance (Ichida 1999).

The first reported mutation was in the gene tafazzin (*TAZ*) which encodes a novel protein family with unknown function to date (Bleyl 1999). Mutations in α-dystrobrevin (*DTNA*), ZO-2 Associated Speckle Protein (*ZASP*), and sarcomere-encoding genes like β-myosin heavy chain (MYH7), α-cardiac actin (*ACTC1*), and cardiac specific troponin (*cTnT*) have been reported. In addition, sodium channel gene mutations (*SCN5A*) and cytoskeletal protein dystrophin mutations encoded by *DMD* gene have also been seen (Towbin 2010). There has been few mutations reported by this rare type of cardiomyopathy and further research should focus to identify possible causative agents of the disorder (Figure 3).

4. Complications

Arrhythmias, including supraventricular tachycardia, VT, and VF are commonly seen in all subtypes of LVNC. Patients are also known to develop systemic arterial embolism and conduction abnormalities like sinus bradycardia or complete heart block. Wolff-Parkinson-White syndrome which is a disorder characterized by problems in the electrical system of the heart is also seen to be associated with LVNC (Ichida 1999). The disorder presents in males with neutropenia, 3-methylglutaconic aciduria, and mitochondrial abnormalities (Kelley 1991). SCD due to coronary heart failure is common along with sepsis due to leukocyte dysfunction. Endocardial fibroelastosis may be prominent with abnormal mitochondria in shape and quantity (Towbin 2010).

5. Therapy and Prevention

Similar to the case of other cardiomyopathies, ACE inhibitors such as captopril along with β-adrenergic blocking agents such as metoprolol are given to patients with systolic dysfunction and heart failure. The subtype close to HCM phenotype is given only β-adrenergic agents only. Patients with mitochondrial or metabolic abnormalities are given vitamins along with coenzyme Q10, carnitine, riboflavin, and thiamine. LVNC patients associated with CHD are considered for pacemakers, implantable defibrillators, and intracardiac ablations (Towbin 2010).

F. Next Generation Sequencing in Cardiomyopathy

Medical and clinical management is shifting from symptomatic treatment to genomic studies to reveal the underlying cause of the disease. Mutations were studied for long time using Sanger sequencing, yet due to its limitations and the demand for quick, massive and cost effective DNA sequencing, next generation sequencing (NGS) was introduced. NGS has made it possible by massive parallel sequencing to generate huge amount of data in a short time. The use of NGS is shifting the medical field towards personalized medical management and approach for every disease (Miller 2013). NGS is ability to sequence millions of DNA fragments by massive parallel sequencing. Sequencing technologies are basically differentiated on the basis of template preparation, sequencing, imaging, and data analysis. Thus the first step is template preparation in which DNA libraries are formed and amplified either by PCR through multiplexing approach using multiple primers or through hybrid capture. Sequences are obtained after subjecting amplified libraries into sequencers, where different sequencing approaches are employed. Data analysis follows where raw data is processed by removing adapters and low quality reads. Quality reads are then compared to reference genome and bioinformatics tools come into play (Biswas 2014).

Cardiomyopathies, as discussed extensively before, have a familial occurrence of 20-30% (Maron 2006). Traditional gene mapping have identified some candidate genes, yet a huge number of patients remained unidentified with known genetic causes. This required deep re-sequencing with extensive genomic region coverage, opening the doors for NGS application to the cardiomyopathy disease. Studies have focused on whole exome and targeted candidate gene sequencing of proband with affected and unaffected family

members. In a clinical setup, the panels of cardiomyopathy candidate genes are presented as targeted sequences panels used as diagnostic tools. Researchers, however, are shifting to whole exome sequencing. With a reduced cost, it is providing a wider picture along with the possibility of discovering novel genes that may implicate new therapeutic pathways. The usage of NGS technologies nowadays in cardiomyopathy research studies and in diagnostic clinical setups helps researchers and clinicians identify pathways and mechanisms leading to disease in attempt for a definitive and promising therapy (Wells 2013, Campbell 2013).

G. Aim of the Study

Though the genetics behind this complication is well established, making use of the genetic data to manage the disease is still facing many challenges. One of the encountered hurdles is the burden the physicians, genetic counselors, and family members face when dealing with the results of the genetic screening. Additionally, the difficulty of determining the treatment or follow-up regimen that should be adopted in order to prevent SD of patients who are found to be genotype positive for the discovered mutations and yet phenotype negative and, thus, who have no clinical disease. In the Europeans and African-Americans populations, genetic studies have been conducted extensively focusing on genetic susceptibility for cardiomyopathies. Middle Eastern populations, however, have been only rarely studied. Screening of mutations in a panel of genes that are responsible for the majority of the cardiomyopathies frequently diagnosed in patients admitted to the American University of Beirut Medical Center in Lebanon will aid in establishing an

efficient genetic counseling coupled with a broader understanding of the genotype/phenotype correlations. Our specific aims are:

1- Perform genetic screening for mutations for patients in a panel of genes implicated in cardiomyopathy phenotypes using next-generation “targeted” sequencing

2- Perform genetic screening for patients using next-generation whole exome sequencing

CHAPTER II

MATERIALS AND METHODS

A. Sample Collection

Patients presenting for *indicated* echocardiography referred to the pediatric or echocardiography labs at the AUBMC were addressed by the study coordinator concerning the study after finishing their echo either in their rooms or at the study staff offices (for outpatients). Potential subjects, with diagnosed disease or suspicious to have cardiomyopathy, of any age were recruited under the patient group in the study.

Inclusion Criteria:

1- All ages were eligible for enrollment

2- Documented echo diagnosis of hypertrophic, dilated, restrictive, arrhythmogenic right ventricular dysplasia, and non-compaction or hyper-trabeculated cardiomyopathies.

Exclusion Criteria:

1- General medical illness that could interfere with the diagnostic accuracy

2- Presence of other cardiac disorders or suspected acquired cardiomyopathy disease

We set-up a clinical-pathological database and a biological repository (DNA) for all patients diagnosed with cardiomyopathy. Informed consent were obtained from participants for DNA isolation. Specific information was gathered *via* a data collection sheet.

B. DNA Extraction

Blood samples were obtained by venipuncture, shipped to AUB where genomic DNA was extracted from peripheral blood lymphocytes (Qiagen, Hilden, Germany). Extraction of DNA starts by adding 200 μL of protease, 2.4 mL of Lysis buffer and 2 mL of patient's blood. Tubes are mixed and inverted 15 times then vortexed for 1 minute. They are kept in water bath at 70°C for 10 minutes. Then we add 2 mL of ethanol and we mix and invert the tubes and vortex for 30 seconds. Half of the volume (=3 mL) is put in a filter falcon and centrifuged for 3 minutes at 3000 revolutions per minute (rpm) at 15 °C. We discard the solution and add the remaining part and centrifuge again. Add 2 mL washing buffer 1 then centrifuge for 4 minutes at 4700 rpm at 15°C. Then add 2 mL washing buffer 2 then centrifuge for 20 minutes at 15°C. Air dry for 5 minutes, then transfer to a new falcon. Add 180 μL elution buffer and wait for 5 minutes at room temperature. We finally centrifuge at 4700 rpm for 5 minutes.

Genotyping was performed blinded to clinical status. DNA samples were quantitated with a Nanodrop spectrophotometer (ND-1000) and normalized to a concentration of 50 ng/μL (diluted in 10 mM Tris/1 mM EDTA). Samples were coded and stored at -80°C. Culture and transformation of peripheral blood lymphocytes from consented candidate individuals were processed in media enriched with EBV-containing solution. Cell transformation allows cells to bypass the normal replicative senescence phase and become immortal. The immortalized cell lines thus obtained were stored in liquid nitrogen, ready to be recovered and harvested for DNA extraction and mutation analysis. This ensured a permanent source of cells and DNA, sparing patients the discomfort of repeated collections.

C. Haloplex Next Generation Targeted Sequencing

1. Digestion of genomic DNA with restriction enzymes

Targeted exome sequencing was performed on 10 patients using Haloplex Target Enrichment System by Agilent Technologies (Santa Clara, United States). In the fist step, genomic DNA (gDNA) samples are digested by 16 different restriction enzymes to create a library of gDNA restriction fragments. We prepared the DNA samples for the run. For 12-reaction runs, we prepare 11 gDNA samples and one Enrichment Control DNA (ECD) sample. In separate 0.2-mL PCR tubes, we diluted 225 ng of each gDNA sample in 45 µL nuclease-free water, for a final DNA concentration of 5 ng/µL. This was stored on ice. In a separate 0.2-mL PCR tube, we dispensed 45 µL of the supplied Enrichment Control DNA (ECD). Then we stored on ice. The gDNA was digested in eight different restriction reactions, each containing two restriction enzymes. The 16 restriction enzymes (RE) are provided in two 8-well strip tubes that are distinguished by red and green color markers. Enzymes are combined from corresponding wells of the red- and green-marked strip tubes, along with restriction buffer and bovine serum albumin (BSA) to make eight different RE Master Mixes (Figure 6).

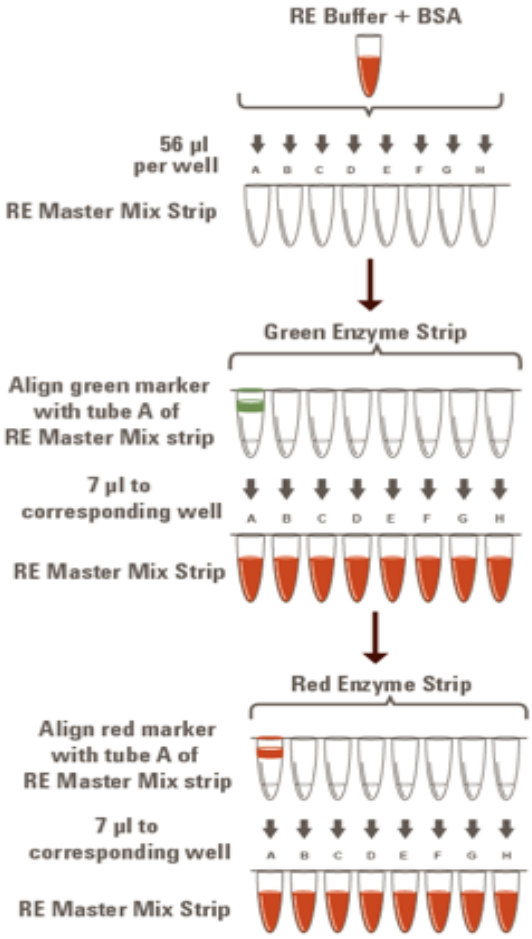


Figure 6. Preparation of the restriction enzyme master mix strip for 12-sample run.

For 12 reactions, we combined 476 µL of RE buffer with 11.9 µL BSA solution in 1.5 mL tube. We mixed by vortexing briefly. In an 8 well tube labeled A to H, we dispensed 56 µL of the RE buffer/BSA mixture in each tube. Using a multichannel pipette, we added 7 µL of the RE enzyme from the green enzyme strip provided with the kit and 7 µL of the RE enzyme from the red enzyme strip. This mixture was mixed by gentle vortexing and then span briefly. We aligned the RE Master Mix strip along the vertical side of a 96-well PCR

plate provided by Biorad (Hercules, Calofornia). Using a multichannel pipette, we carefully distributed 5 µL of each RE master mix row-wise into each well of the plate. Each row of the 96-well plate would contain 5 µL per well of the same RE combination (Figure 7). Then the DNA samples were aligned along the horizontal side of the digestion reaction plare and we distributed 5 µL of DNA samples column-wise into each well of the plate. The plate was sealed thoroughly with adhesive plastic film. We vortexed the plate to mix the digestion reaction and then briefly span the plate in a plate centrifuge. Wells of the prepared 96-well plate contained complete 10-µL restriction digestion reactions. In this format, each column corresponds to one DNA sample digested in eight different restriction reactions. We placed the Restriction Digest Reaction Plate in a thermal cycler (T-100 thermal cycler with gradient-Biorad) and it was run on 37°C for 30 minutes and 8°C hold time.

Figure 7. Restriction digestion reaction plate

2. Validation of the Restriction Digestion by Electrophoretic Analysis

The Restriction Digest Reaction Plate was kept on ice during validation. We transferred 4 μL of each ECD digestion reaction from wells of the digestion reaction plate to fresh 0.2-mL PCR tubes. We incubated the removed 4 μL samples at 80°C for 5 minutes to inactivate the RE. We analyzed the prepared samples using gel electrophoresis. We prepared 1.5% agarose gel where we weighed 4.5 g of agarose in 300 mL TBE Sample Buffer. We prepared an undigested DNA gel control by combining 2 μL of the ECD stock solution and 2 μL of nuclease-free water. We added 1 μL loading dye (5X) (Biorad) to each 4-μL ECD sample and loaded 5 μL of each sample on the gel. In one adjacent lane, we loaded 200 ng of a 50-bp DNA ladder (Biorad). We ran the gel at 210 V for approximately 15 minutes and stained it in Ethidium Bromide Stain and we visualized bands under UV radiation.

3. Hybridization of digested DNA to HaloPlex probes for target enrichment and sample indexing

In this step, the collection of gDNA restriction fragments is hybridized to the HaloPlex probe capture library. HaloPlex probes are designed to hybridize selectively to fragments originating from target regions of the genome and to direct circularization of the targeted DNA fragments. During the hybridization process, Illumina sequencing motifs including index sequences are incorporated into the targeted fragments. We prepared a Hybridization Master Mix by combining 650 μL of the hybridization solution and 260 μL of the HaloPlex probe. We distributed 70 μL of the Hybridization Master Mix to each of 12 0.2 mL tubes. We added 10 μL of the appropriate Indexing Primer Cassette to each tube

containing Hybridization Master Mix. We transferred digested DNA samples from the 96-well Restriction Digest Reaction Plate directly into the hybridization reaction tubes. For the ECD sample, we added 32 µL of nuclease-free water, in addition to the digested DNA samples, to compensate for the volume removed for digest validation. We vortexed the mixtures briefly and then span tubes briefly. We placed the hybridization reaction tubes in a thermal cycler (T-100 thermal cycler with gradient-Biorad) and ran the appropriate program in Table 1.

Step	Temperature	Cancer Research Panel or Cardiomyopathy Research Panel
Step 1	95°C	10 minutes
Step 2	54°C	3 hours

Table 1. Thermal cycler program for HaloPlex probe hybridization

4. Capturing of the Targeted DNA

In this step, the circularized target DNA-HaloPlex probe hybrids, containing biotin, were captured on streptavidin beads. We started by removing the Capture Solution, Wash Solution, Ligation Solution and SSC Buffer from -20°C and the HaloPlex Magnetic Beads form + 4 °C. We prepared 2M acetic acid fresh and 50 mM NaOH for use in the DNA elution step. We vigorously re-suspended the provided HaloPlex Magnetic Beads on a

vortex mixer. The magnetic beads settle during storage. We transferred 520 μL of HaloPlex Magnetic Bead suspension. We put the tube into a 1.5 mL tube-compatible magnetic rack for 5 minutes. After verifying that the solution has cleared, we carefully removed and discarded the supernatant. We added an equivalent volume of Capture Solution to the beads and resuspended by pipetting up and down. We removed the hybridization reactions from the thermal cycler and immediately added 40 μL of the prepared bead suspension to each 160-μL hybridization reaction. After adding the magnetic beads, mixed the capture reactions thoroughly by pipetting up and down 15 times. We incubated the capture reactions at room temperature for 15 minutes. We briefly span the tubes in a desktop centrifuge and then transferred the tubes to Agencourt SPRIPlate Super Magnet magnetic plate. We waited for the solution to clear (about 30 seconds), then removed and discarded the supernatant. Then we washed the bead-bound samples: by removing the capture reaction tubes from the magnetic plate and adding 100 μL of Wash Solution to each tube. We resuspended the beads thoroughly by pipetting up and down 10 times using a 100 μL multichannel pipette. We incubated the tubes in a thermal cycler at 46°C for 10 minutes, using a heated lid. We briefly span the tubes in a desktop centrifuge at room temperature and then transfer the tubes to the magnetic plate. We waited for the solution to clear (about 30 seconds), then carefully removed and discarded the supernatant.

5. Ligation of the captured, circularized fragments

We prepared a DNA ligation Master mix by combining 617.5 μL of ligation solution and 32.5 μL of DNA ligase. We added 50 μL of the DNA ligation master mix to the beads in each DNA capture reaction tube. We resuspended the beads thoroughly by

pipetting up and down 15 times. We incubated the tubes in a thermal cycler at 55°C for 10 minutes, using a heated lid. During the 10-minute incubation, we prepared the PCR master mix.

6. Elution of the Captured DNA with NaOH

When the 10-minute ligation reaction period was complete, we briefly span the ligation reaction tubes in a desktop centrifuge and then transferred the tubes to the magnetic plate. We waited for the solution to clear (about 30 seconds), then carefully removed and discarded the supernatant. We removed the tubes from the magnetic plate and added 100 µL of the SSC Buffer provided with the kit to each tube. We resuspended the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette. We briefly span the tubes and then returned the tubes to the magnetic plate. We waited for the solution to clear (about 30 seconds), then carefully removed and discarded the SSC Buffer using a multichannel pipette. We added 25 µL of 50 mM NaOH, which was freshly-prepared before, to each tube. We resuspended the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette. We incubated the samples for 1 minute at room temperature to allow elution of the captured DNA. Then, we briefly span the tubes and then transfer the tubes to the magnetic plate.

7. PCR Amplification of the Captured Target Libraries

We prepared the following PCR master mix by combining the reagents in Table 2. We mixed the master mix components by gentle vortexing, then distributed 30-µL aliquots to fresh 0.2-mL reaction tubes.

Reagent	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Nuclease-free water	16.1 µL	209.3 µL
5X Herculase II Reaction Buffer	10 µL	130 µL
dNTPs (100 mM, 25 mM for each dNTP)	0.4 µL	5.2 µL
Primer 1 (25 µM)	1 µL	13 µL
Primer 2 (25 µM)	1 µL	13 µL
2 M Acetic acid	0.5 µL	6.5 µL
Herculase II Fusion DNA Polymerase	1 µL	13 µL
Total	**30 µL**	**390 µL**

Table 2. Preparation of PCR master mix

We prepared amplification reactions by transferring 20 µL of cleared supernatant from each tube on the magnetic plate to a PCR Master Mix tubes. We mixed by gentle vortexing and then span briefly to collect the liquid. We placed the amplification reaction tubes in a thermal cycler and ran the program in Table 3.

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	Obtain cycle number from Certificate of Analysis	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	8°C	Hold

Table 3. HaloPlex post-capture DNA amplification PCR program

8. Purification of the Amplified Target Libraries

In this step, the amplified target DNA is purified using AMPure XP beads (Imagen, Iran). We let the AMPure XP beads come to room temperature for at least 30 minutes. We prepared 400 µL of 70% ethanol per sample. Then we transferred 40 µL of each PCR reaction sample to a fresh 0.2-mL tube. We mixed the AMPure XP bead suspension well, until the suspension appears homogeneous and consistent in color. For each sample to be purified, we prepared a bead mix by combining 40 µL of nuclease-free water and 100 µL of the homogenous AMPure XP bead suspension. This was mixed well, until the bead mix suspension appears homogeneous and then we added 140 µL of the homogenous bead suspension prepared to each 40-µL amplified library sample. Using this bead-to-sample volume ratio is imperative to ensure optimal purification results. Samples were incubated for 5 minutes at room temperature with continuous shaking then span briefly to collect the liquid, then placed in the magnetic plate. We waited for the solution to clear (approximately

5 minutes). While keeping the tubes in the magnetic plate, we removed and discarded the cleared solution from each tube using a 200-µL pipette. The beads were not touched while removing the solution. The tubes continued to be kept in the magnetic plate while we added 200 µL of 70% ethanol. We waited for 30 seconds to allow any disturbed beads to settle, then we removed the ethanol using a 200 µL pipette. This step was repeated twice. The tubes were air dried with open lids at room temperature until the residual ethanol completely evaporates. The tubes were removed from the magnetic plate and 40 µL of 10 Tris-HCl buffer (pH 8.0) were added to each sample. They were mixed thoroughly by pipetting up and down 15 times and incubated for 2 minutes at room temperature to allow elution of DNA. Then the tubes were put in the magnetic plate and left for 2 minutes. Finally the clear supernatant (approximately 40 µL) was removed to a fresh tube.

9. Validation and Quantification of Enriched DNA

Using Experion DNA 1K Analysis kit (Biorad, Hercules, Calofornia), we equilibrated kit reagents to room temperature: ST stain, B loading buffer, L ladder and G gel. Then the Gel-Stain (GS) Solution was prepared. 12.5 µL from the ST tube were taken and put in the 250 µL G tube. This new mix was vortexed for 10 seconds, then all the solution was transferred to a spin filter tube. This was centrifuged at 2,400 g for 15 minutes. The filter was then discarded. The next step was to prime the chip on the Experion machine. We added the GS solution to the priming well and C3 is selected on the priming station. Then, 9 µL of the filtered GS solution were pipetted into the other 3 GS wells. 5 µL loading buffer were pipetted into the L well and each sample well, then 1 µL DNA 1K ladder into the well L. We pipet 1 µL of DNA sample into each of the 11 sample wells and

1 μL of DNase free water into any unused well. We placed the chip in the Experion vortex station for 1 minute. Then we run the chip in the Experion electrophoresis station within 5 minutes of loading.

10. Pooling of Samples with Different Indexes for Multiplexed Sequencing

To pool samples effectively, all should be equimolar and have a concentration of 2 nM. All samples were diluted to a 2 nM concentration and an equi-volume was taken from each sample to be pooled and we proceeded to denaturing the libraries. For this purpose, we followed Illumina (San Diego, California) manual for preparing libraries for sequencing on the MiSeq®. We started by preparing a fresh dilution of 0.2N NaOH by combining 10 μL stock 2N NaOH in 90 μL nuclease free water. Then added 5 μL f the 2nM pooled library with 5 μL of the freshly prepared 0.2N NaOH. We vortexed briefly and centrifuged at 280xg for one minute. Incubate for five minutes at room temperature. Dilute the library to 20 pM by adding 20 μL of the 2nM denatured library with 980 μL of pre-chilled Hybridization Buffer (HT1). Then we performed a second dilution to 12.5 pM library where we added 375 μL of the 20 pM library with 225 μL pre-chilled HT1. We kept the sample on ice. The denaturation of the PhiX control provided by the kit was done following the same procedure as the pooled sample denaturation. Then we combined 6 μL of the denatured and diluted PhiX control with 594 μL of the denatured and diluted sample library. This was loaded onto the MiSeq reagent cartridge and put on the machine.

D. SureSelect Next Generation Whole-Exome Sequencing

The samples were prepared according to an Agilent SureSelect Target Enrichment kit preparation guide (Santa Clara, United States) similar to Haloplex preparation. Briefly, we started by a gDNA where a sheering step produces small fragments. We prepared libraries with sequencer specific adaptors and indexes. We hybridized samples with biotinylated RNA library baits. Baits are oligonucleotides that retrieved specific RNA species or genomic DNA fragments of interest for sequencing. The desired DNA or RNA molecules hybridized with the baits, and others did not. Agilent uses ultra long 120mer RNA baits for the highest specificity. We selected targeted regions using magnetic streptavidin beads. The libraries were amplified and sequenced with Illumina HiSeq 2000/2500 sequencer (Figure 8).

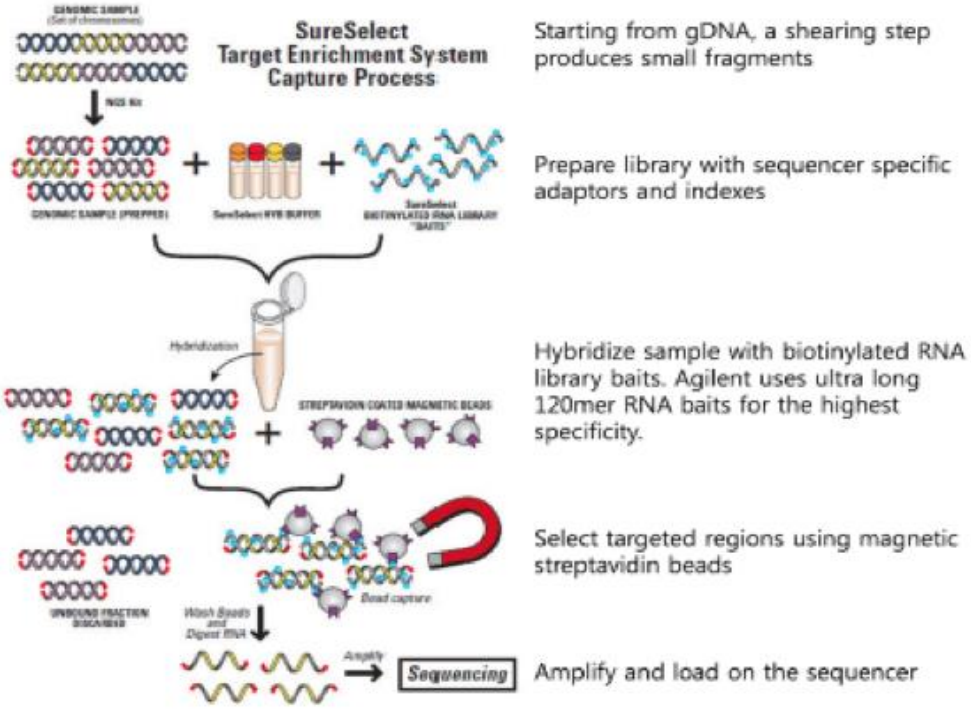


Figure 8. SureSelect target enrichment workflow

E. Data Analysis

Data were analyzed by SureCall software provided by Agilent Technologies (Santa Clara, United States). The database results were compared to was Exac (exac.broadinstitue.org). Novel mutations were evaluated with PolyPhen 2 software (http://genetics.bwh.harvard.edu/pph2/). PolyPhen 2 (Polymorphism Phenotyping v2) is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.

CHAPTER III

RESULTS

A. Clinical Characteristics of the Patients

Patients presenting for *indicated* echocardiography at the AUBMC were consented for blood withdrawal and medical history abstraction. The clinical characteristics of our 26 patients are presented in Table 4 where we had a higher percentage of males over females presenting with cardiomyopathies (Males = 62%, females = 38%). The mode age was 46 years old and 31% had ICD implantation as an effective method of management. Only 27 % had a positive family history of affected siblings or cousins but not parents.

Patient	***Gender***	***Age***	***ICD***	***Family History***	***Sequencing***
EGY-1	M	28	YES	YES	SureSelect
MR 22	F	10	NO	NO	SureSelect
MR 37	M	41	YES	NO	SureSelect
MR 38	F	35	YES	NO	SureSelect
MR 39	M	48	NO	YES	SureSelect
MR 40	M	50	NO	YES	SureSelect
MR 41	M	57	YES	NO	SureSelect
MR 29	M	57	YES	NO	Haloplex
MR 36	F	46	Transplant	YES	Haloplex
MR 42	F	34	NO	NO	Haloplex
MR 45	F	46	YES	NO	Haloplex
MR 47	F	48	NO	NO	Haloplex
MR 49	M	46	YES	NO	Haloplex
NM 1215	M	3	NO	NO	Haloplex
NM 1158	M	19	NO	NO	Haloplex
MR 50	M	47	NO	NO	Haloplex

MR 51	F	51	NO	NO	Haloplex
MR 1	M	NA	NA	NO	Haloplex
MR 3	M	76	NO	NO	Haloplex
MR 5	F	30	NO	YES	Haloplex
MR 6	M	NA	NO	YES	Haloplex
MR 8	F	2	NO	YES	Haloplex
MR 9	F	NA	NA	NO	Haloplex
MR 10	M	36	YES	NO	Haloplex
MR 16	M	8	NO	NO	Haloplex
MR 21	M	53	NO	NO	Haloplex
Results	M: 62% F: 38%	46	31%	27%	NA

Table 4. Clinical characteristics of the patients revealed a higher percentage of men presenting with cardiomyopathy with a mode age of 46. A reasonable percentage of patients were managed by ICD implantation. The number of patients with family history of cardiomyopathy was low. (M=male, F=female, NA=not applicable)

B. Panel of Genes of Cardiomyopathy

The SureSelect method by Illumina (San Diego, California) was used for sequencing on seven chosen patients infers whole exome sequencing, however the Haloplex method by Agilent (Santa Clara, United States) used assumes targeted sequencing against 34 genes implicated in cardiomyopathy presented in Table 5.

GENE	***NAME***	***CHROMO-SOME***	***CARDIO-MYOPATHY***
ABCC9	ATP-binding cassette, sub-family C member 9	12	DCM
ACTN2	Alpha-actinin 2	1	DCM
CSRP3	Cysteine and glycine-rich protein 3 gene	11	DCM, HCM
DES	Desmin gene	2	DCM, RCM
DSC2	Desmocollin-2	18	HCM
DSG2	Desmoglein-2	18	DCM
DSP	Desmoplakin	6	DCM, ARVC

JUP	Junction plakoglobin	17	ARVC
LDB3	LIM domain binding 3	10	LVNC
LMNA	Lamin A/C	1	DCM
MYBPC3	myosin-binding protein C cardiac-type	11	DCM, HCM
MYH6	Myosin heavy chain alpha	14	DCM
MYH7	Myosin heavy chain beta	14	DCM, HCM, RCM
MYL2	Myosin regulatory light chain 2	7	HCM
MYL3	Myosin essential light chain	3	HCM
MYOZ2	Myozenin-2	4	HCM
NEXN	Nexilin	1	DCM, HCM
OPN4	Melanopsin	10	DCM
PKP2	Plakophilin-2	12	ARVC
PLN	Phospholamban	6	DCM, HCM
RBM20	RNA-binding protein	10	DCM
SCN5A	Sodium channel voltage gated type 5	3	DCM
SGCD	Delta-sarcoglycan	5	DCM
TAZ	Tafazzin	X	DCM
TCAP	Telethonin	17	DCM, HCM
TGFB3	Transforming growth factor beta-3	14	ARVC
TMEM43	Transmembrane protein 43	3	ARVC
TNNC1	Troponin C	3	DCM
TNNI3	Troponin I cardiac muscle	19	DCM, HCM, RCM
TNNT2	Cardiac Troponin T	1	DCM, HCM, RCM
TPM1	Tropomyosin alpha-1 chain	15	DCM, HCM, RCM
TTN	Titin	2	DCM, HCM
TTR	Transthyretin	18	RCM
VCL	Vinculin	10	DCM, HCM

Table 5. Panel of genes used by Haloplex sequencing implicated in cardiomyopathy

C. Incidence of Cardiomyopathy Types in the Population

Patients presenting to our facilities at the American University of Beirut Medical Center are from different parts of Lebanon and the Middle East and our sample population reflects the percentages of cardiomyopathy types seen. HCM came out to have the highest incidence among our patients with a 48%, followed by DCM with 20%. ARVC and RCM had similar incidence with 17% and 12% respectively. LVNC as expected had a very low incidence of occurrence of 3%. Data presented in Figure 9.

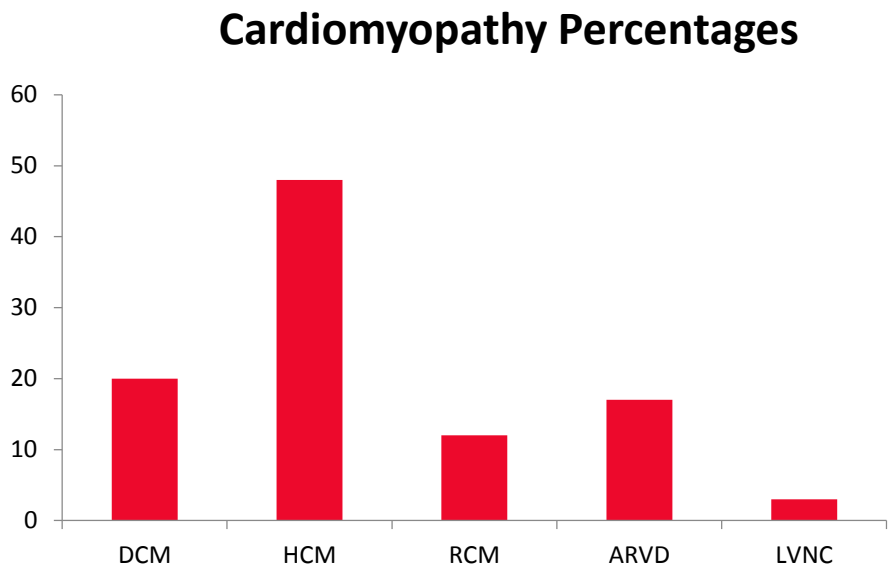


Figure 9. Incidence of cardiomyopathy types in the sample population that revealed a high percentage of occurrences of HCM with 48%. DCM was seen in 20% of patients followed by the three less occurring types of RCM, ARVC, and LVNC with 17 %, 12%, and 3% respectively.

D. Quality Control of Our Runs

The quality control of the runs performed are presented as per the Surecall software with a QC>200 and read depth for each fragment >30. This is shown in Figures 10 and 11.

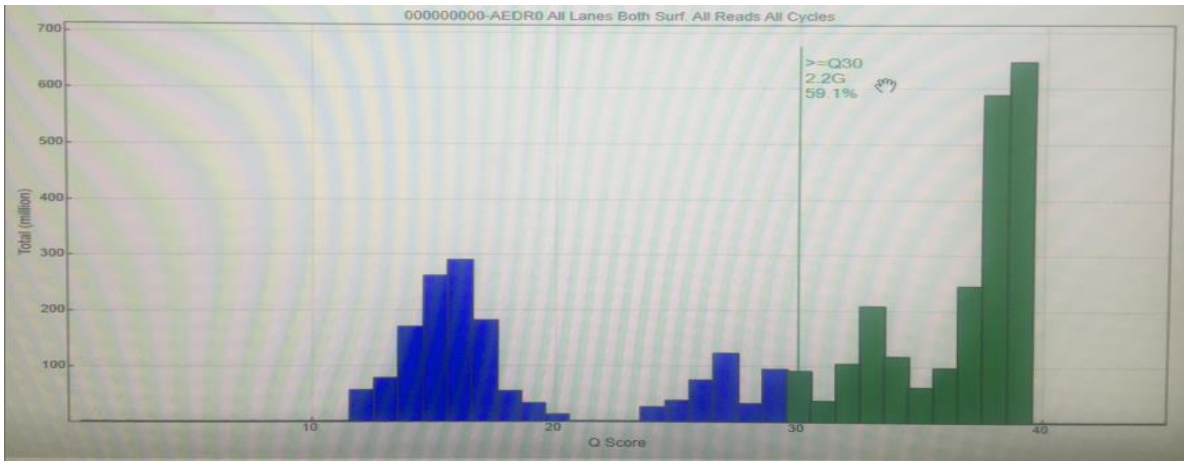


Figure 10. Quality control of run 1

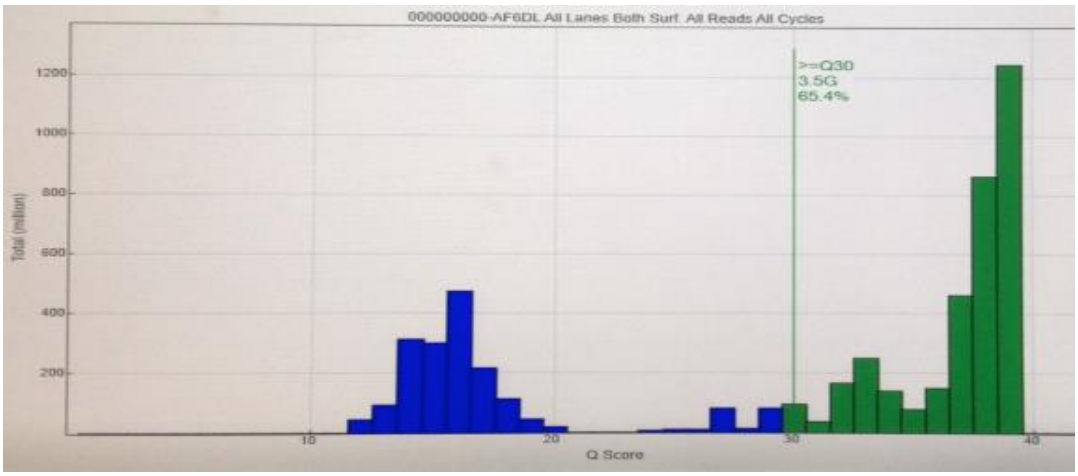


Figure 11. Quality control of run 2

E. Digestion Validation of Bands by Gel Electrophoresis

The ECD sample contains genomic DNA mixed with an 800-bp PCR product that contains restriction sites for all the enzymes used in the digestion protocol. When analyzing validation results, the undigested control had gDNA bands at >2.5 kbp and a PCR product band at 800 bp. Each of the eight digested ECD samples had a smear of gDNA restriction fragments between 100 and 2500 bp, overlaid with three predominant bands at approximately 125, 225 and 450 bp. These three bands correspond to the 800-bp PCR product-derived restriction fragments, and precise sizes will differ after digestion in each of the eight RE master mixes.

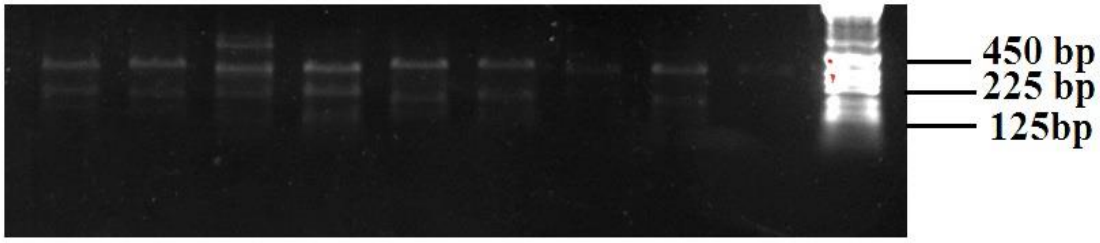
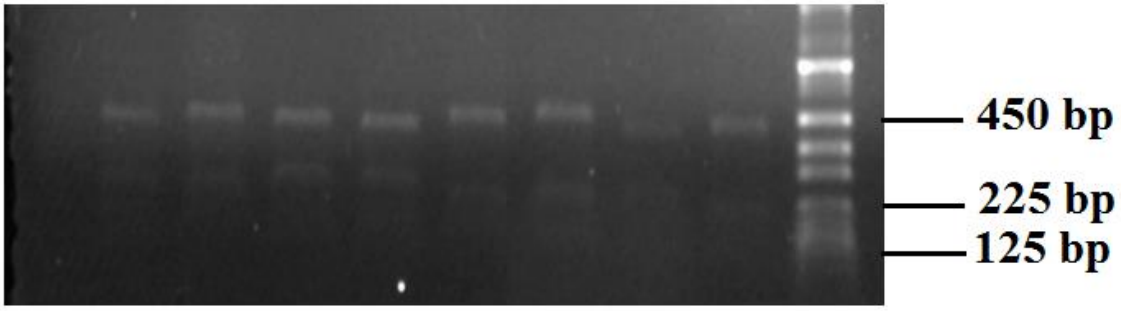


Figure 12: Gel digestion validation by electrophoresis- First run

Figure 13: Gel digestion validation by electrophoresis-Second run



F. Genetic Results

1. Haloplex: Targeted Sequencing

The generated Fastq files (50 bp paired-end) were aligned with Novoalign (www.novocraft.com). Bam files were processed with the Genome Analysis Toolkit (GATK), version 2.3–9, and Surecall (http://www.genomics.agilent.com) was used to call variants and annotate them. Only variants that are covered 10X, that are of high quality scores, and that are rare (minor allele frequency, <1% in both the analyzed dataset and the Exome Sequencing Project:http://exac.broadinstitute.org/) were selected. The variants were arranged in 4 categories according to their potential effects on protein structure and function with Category I being the most severe and Category IV the least severe. Category I regroups non-sense and frameshift variants, Category II missense variants, Category III synonymous variants, and Category IV intronic variants. Filtering of results for the 19 patients was done, first by choosing categories I and II, second by choosing "Downstream Modifier Coding" or "Non-Synonymous-Coding" in the Effect and Primary Effect category. They are presented in the tables below (Tables 6- 24). Since we did not have any

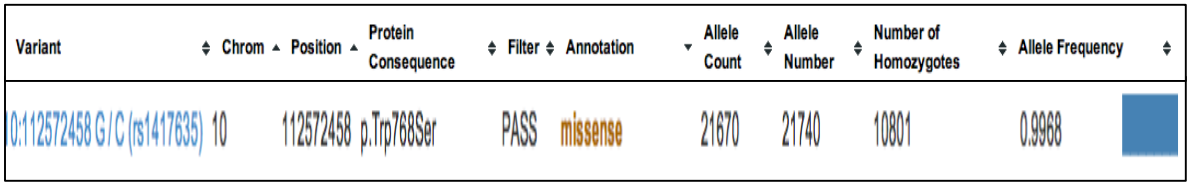
patient history whereby the parents were affected, we focused on homozygous variants screening and considered them as potential disease-causing variants. The variants were compared to Exac (Figure 13 shown as an example) database which gives the prevalence of the variant among the normal population, and we used the Poly-Phen 2 software to predict the severity of the variation effects on the function of the protein.

The first patient in this Haloplex sequencing category is MR 29 that showed several homozygous variants in the form of non-synonymous missense variants in *TTN*, *RBM20* in 2 different positions, *PKP2* also in 2 positions, *JUP*, *NEXN*, *SCN5*A, *DSP*, and finally *TGFB3* in 2 positions. Several were novel variants (*NEXN* at position 78392204, *SCN5A* at position 38640426, *DSP* at position 7585195, and *TGFβ3* at positions 76425544, 76425549, and 76425550). The effect of the latter on the clinical setting of cardiomyopathy was evaluated by PolyPhen 2 and the results showed a probable destructive mutation in *TGFβ3* in the 3 positions. When comparing patient MR 29 to other patients on which Haloplex sequencing was performed, we noted the high prevalence of some homozygous variants among patients. *RBM20* variant at position112572458 was repeated in patients MR36, MR29, MR42, MR45, MR47, MR49, NM1215, NM1158, MR50, MR51, MR5, MR6, MR10, and MR16 as the results show. Exac showed high allele frequency (a number close to 1) in the normal population suggesting that this variant is non-disease causing. In addition its high prevalence in our study sample assures this hypothesis. Also *RBM20* variant in position 112595719 was seen in patients MR29, MR42, MR45, MR49, NM1215, NM1158, MR50, MR51, MR5, MR6, MR10, and MR16. Similarly, *JUP* variant at position 39912145 was prevalent in MR36, MR29, MR42, MR47, MR49, NM1215, NM1158, MR50, MR51,MR1, MR3, MR5, MR6, MR10, MR16,and MR21. *TTN* variants as well at

several positions was highly prevalent in our patients as shown in the tables and this tells that all those variant even if present in a homozygous state, they are non-disease causing due to their high abundance.

MR 29	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
TTN		x		C	T	179545859	HET	
TTN		x		T	C	179644855	HOM	High freq
TTN		x		G	A	179650408	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
PKP2		x		A	G	33021934	HOM	0.18
PKP2		x		C	A	33049457	HOM	0.0243
MYH6		x		A	G	23861811	HET	
MYH6		x		C	T	23876267	HET	
JUP		x		T	A	39912145	HOM	0.6592
DSG2		x		A	G	29104714	HET	
NEXN		x		A	G	78392204	HOM	NOT FOUND
SCN5A		x		A	C	38640426	HOM	NOT FOUND
DSP		x		T	A	7585195	HOM	NOT FOUND
TGFβ3		x		A	G	76425544	HOM	NOT FOUND
TGFβ3		x		T	C	76425549	HOM	NOT FOUND
TGFβ3		x		T	G	76425550	HOM	NOT FOUND

Table 6. MR29 Haloplex sequencing results showing several homozygous variants which were evaluated on Exac to check their novelty and significance. PolyPhen 2 shows TGFβ3 as a probably damaging mutation.



Variant	Chrom	Position	Protein Consequence	Filter	Annotation	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
10:112572458 G/C (rs1417635)	10	112572458	p.Trp768Ser	PASS	missense	21670	21740	10801	0.9968

Figure 14: Exac result for *RBM20* variant at position 112572458 showing a high allele frequency of 0.9968 and suggesting that this variant is non disease causing.

MR 36	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
MYH7		Exon splicing		T	G	23885523	HOM	NOT FOUND
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HET	
JUP		x		T	A	39912145	HOM	0.6592

Table 7. MR36 Haloplex sequencing results showing a novel variant of *MYH7* on position 23885523.

MR 42	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
TTN		x		C	T	179623758	HET	
TTN		x		C	T	179629461	HET	
TTN		x		T	C	179632496	HET	
TTN		x		C	T	179638721	HET	
TTN		x		G	A	179644035	HOM	High Freq
TTN		x		T	C	179644855	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
MYBPC3		x		T	C	47370041	HET	
PKP2		x		A	G	33021934	HET	

MYH6		x		G	A	23863291	HET	
JUP		x		T	A	39912145	HOM	0.6592
TNNI3		x		C	T	55665410	HOM	0.0659

Table 8. MR42 Haloplex sequencing results show several homozygous variants highlighted in red yet all of no clinical significance due to their high allele frequency in the population.

MR 45	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense					
LMNA		x		T	C	156105028	HET	
LMNA		x		T	C	156106185	HET	
TTN		x		C	T	179545859	HET	
TTN		x		C	T	179623758	HOM	High Freq
TTN		x		C	T	179629461	HOM	High Freq
TTN		x		G	A	179644035	HOM	High Freq
TTN		x		T	C	179644855	HOM	High Freq
TTN		x		G	A	179650408	HET	
TMEM43		x		A	T	14174427	HOM	0.3209
TMEM43		x		T	C	14175262	HOM	0.3724
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
MYBPC3		x		T	C	47370041	HET	
PKP2		x		A	G	33021934	HET	
PKP2		x		C	A	33049457	HET	

Table 9. MR 45 Haloplex sequencing results show several homozygous variants highlighted in red yet all of no clinical significance due to their high allele frequency in the population.

MR 47	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense					
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
MYH6		x		A	G	23861811	HOM	0.3461
MYH6		x		C	T	23876267	HET	
JUP		x		T	A	39912145	HOM	0.6592

Table 10. MR47 Haloplex sequencing results show several homozygous variants highlighted in red yet all of no clinical significance due to their high allele frequency in the population.

MR 49	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense					
LMNA		x		T	C	156105028	HET	
LMNA		x		T	C	156106185	HET	
TTN		x		C	T	179623758	HOM	High Freq
TTN		x		C	T	179629461	HOM	High Freq
TTN		x		G	A	179644035	HOM	High Freq
TTN		x		T	C	179644855	HOM	High Freq
TTN		x		C	T	179648807	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HET	
PKP2		x		A	G	33021934	HET	
MYH6		x		A	G	23861811	HOM	0.3461
MYH6		x		C	T	23876267	HET	
JUP		x		T	A	39912145	HOM	0.6592

Table 11. MR49 Haloplex sequencing results reveal several homozygous variants highlighted in red yet all of no clinical significance due to their high allele frequency in the population.

NM 1215	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
LMNA		x		T	C	156105028	HET	
TTN		x		C	T	179623758	HOM	High Freq
TTN		x		C	T	179629461	HOM	High Freq
TTN		x		G	A	179644035	HOM	High Freq
TTN		x		T	C	179644855	HOM	High Freq
DSP		x		A	T	7582993	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HET	
MYBPC3		x		G	A	47364146	HET	
MYBPC3		x		T	C	47370041	HET	
MYBPC3		x		T	C	47371330	HET	
MYH6		x		A	G	23861811	HET	
MYH6		x		C	T	23876267	HET	
JUP		x		T	A	39912145	HOM	0.6592
DSC2		x		T	C	28649042	HET	
DSG2		x		A	G	29104714	HET	
TNNI3		x		C	T	55665410	HET	

Table 12. NM 1215 Haloplex sequencing result shows several homozygous variants highlighted in red yet all of no clinical significance due to their high allele frequency in the population.

NM 1158	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
TTN		x		G	A	179474668	HET	
TTN		x		C	T	179623758	HOM	High Freq
TTN		x		C	T	179629461	HOM	High Freq
TTN		x		C	G	179642589	HET	
TTN		x		T	C	179644855	HET	
TTN		x		G	A	179659912	HET	
TTN		x		C	A	179666982	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
PKP2		x		G	T	33030921	HET	
JUP		x		T	A	39912145	HOM	0.6592

Table 13. NM 1158 Haloplex sequencing result shows several homozygous variants highlighted in red yet all of no clinical significance due to their high allele frequency in the population.

MR 50	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
TTN		x		C	T	179623758	HET	
TTN		x		C	T	179628918	HET	
TTN		x		C	T	179629461	HET	
TTN		x		C	T	179634936	HET	
TTN		x		C	G	179637861	HET	
TTN		x		C	T	179641975	HET	
TTN		x		G	A	179644035	HOM	High Freq
TTN		x		T	C	179644855	HET	
TTN		x		G	A	179650408	HET	
TMEM43		x		A	T	14174427	HET	
TMEM43		x		T	C	14175262	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
MYBPC3		x		T	C	47370041	HET	
PKP2		x		A	G	33021934	HET	
JUP		x		T	A	39912145	HOM	0.6592
JUP		x		C	T	39925713	HET	
DSG2		x		A	G	29104714	HET	

Table 14. MR50 Haloplex sequencing result shows several homozygous variants highlighted in red yet all of no clinical significance due to their high allele frequency in the population.

MR 51	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
MYH7		Exon Splicing		T	G	23885523	HOM	NOT FOUND
LMNA		x		T	C	156105028	HET	
LMNA		x		T	C	156106185	HET	
TTN		x		C	T	179623758	HET	
TTN		x		C	T	179629461	HET	
TTN		x		T	C	179632496	HET	
TTN		x		C	T	179638721	HET	
TTN		x		G	A	179644035	HOM	High Freq
TTN		x		T	C	179644855	HET	
TTN		x		G	A	179650408	HET	
SCN5A		x		T	C	38645420	HET	
TNNC1		x		G	A	52488009	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
PKP2		x		A	G	33021934	HET	
PKP2		x		C	A	33049457	HET	
JUP		x		T	A	39912145	HOM	0.6592
TNNI3		x		C	T	55665410	HET	

Table 15. MR51 Haloplex sequencing result shows a novel variant of *MYH7* on position 23885523.

MR 1	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
LMNA		x		C	A	156105738	HOM	NOT FOUND
TTN		x		C	T	179629461	HOM	High freq
TTN		x		G	A	179644035	HOM	High freq
TTN		x		T	C	179644855	HOM	High freq
TMEM43		x		A	T	14174427	HOM	0.3209
TMEM43		x		T	C	14175262	HET	
SCN5A		x		G	A	38592068	HOM	NOT FOUND
SCN5A		x		G	T	38674686	HOM	NOT FOUND
DSP		x		A	T	7565727	HOM	0.02993
DSP		x		A	C	7579966	HOM	NOT FOUND
MYBPC3		x		T	C	47370041	HET	
MYH6		x		C	T	23876267	HOM	0.0698
ACTC1		x		G	A	35084333	HET	
JUP		x		T	A	39912145	HOM	0.6592
DSC2		x		G	A	28654750	HOM	0.00124

Table 16. MR1 Haloplex sequencing result showing a novel variants of *LMNA* on position 156105738, along with *SCN5A* variant on position 38592068 and position 38674686. PolyPhen 2 shows *DSP* at position 7579966 to be probably damaging.

MR 3	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense					
NEXN		x		G	A	78392446	HOM	0.814
LMNA		x		C	A	156106726	HET	
TTN		x		C	T	179623758	HOM	
TTN		x		C	A	179643614	HET	
TTN		x		T	C	179644855	HOM	High Freq
TTN		x		G	A	179650408	HOM	High Freq
DES		x		G	T	220285366	HOM	NOT FOUND
SCN5A		x		C	A	38603989	HOM	NOT FOUND
MYH6		x		A	G	23861811	HOM	0.3461
MYH7		x		G	C	23886409	HET	
TGFβ3		x		C	A	76437907	HOM	NOT FOUND
JUP		x		T	A	39912145	HOM	0.6592

Table 17. MR3 Haloplex sequencing result shows a *DES* novel variant at position 220285366 and *SCN5A* variant at position 38603989. *TGF β3* was interestingly mutated in this patient at position 76437907. All three were evaluated on PolyPhen 2 to check their clinical significance and DES and TGFβ3 reveals to be clinically damaging

MR 5	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
LMNA		x		G	T	156106085	HOM	NOT FOUND
TTN		x		C	T	179629461	HOM	High Freq
TTN		x		G	A	179644035	HOM	High Freq
TTN		x		T	C	179644855	HOM	High Freq
TTN		x		G	A	179650408	HET	
TMEM43		x		A	T	14174427	HOM	0.3209
TMEM43		x		T	C	14175262	HET	
VCL		x		C	A	75842299	HOM	NOT FOUND
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
PKP2		x		A	G	33021934	HET	
MYH6		x		C	A	23854182	HOM	NOT FOUND
MYH6		x		C	G	23858697	HOM	0.00325
MYH6		x		A	G	23861811	HOM	0.3461
MYH7		x		G	C	23886409	HET	
JUP		x		T	A	39912145	HOM	0.6592
DSC2		x		C	T	28648975	HOM	0.0495

Table 18. MR5 Haloplex sequencing result shows *LMNA* novel variant at position 156106085 along with *VCL* at position 75842299. Also interestingly, *MYH6* is mutated at position 23854182. PolyPhen 2 shows *LMNA* to be clinically damaging.

MR 6	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
LDB3			x	C	A	88476514	HOM	NOT FOUND
TTN		x		T	C	179486603	HOM	High freq
TTN		x		C	T	179629461	HOM	High freq
TTN		x		C	A	179643614	HOM	High freq
TTN		x		G	A	179644035	HOM	High freq
TTN		x		G	A	179650408	HOM	High freq

TMEM43		x		T	C	14175262	HET	
VCL		x		G	T	75830737	HOM	NOT FOUND
VCL		x		G	T	75854211	HOM	0.0000009
VCL		x		G	A	75874563	HOM	NOT FOUND
LDB3		x		C	A	88477829	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		C	A	112581552	HOM	NOT FOUND
RBM20		x		G	C	112595719	HOM	0.7637
MYBPC3		x		C	A	47356640	HOM	NOT FOUND
MYBPC3		x		T	C	47371330	HOM	NOT FOUND
ABCC9		x		G	A	22035738	HOM	0.000041
PKP2		x		A	G	33021934	HOM	0.18
MYH6		x		A	G	23861811	HET	
MYH6		x		G	T	23865608	HOM	NOT FOUND
MYH6		x		C	A	23868187	HOM	NOT FOUND
MYH7		x		G	C	23886409	HOM	NOT FOUND
MYH7		x		G	A	23894567	HET	
JUP		x		T	A	39912145	HOM	0.6592
JUP		x		T	G	39925903	HOM	NOT FOUND
JUP		x		T	C	39925906	HOM	NOT FOUND
JUP		x		A	T	39925909	HET	
DSC2		x		G	T	28667676	HET	
DSG2		x		A	G	29104714	HET	

Table 19. MR6 Haloplex sequencing result came to be of high interest due to its significance. Several variants were novel (*LDB3*, *VCL*, *RBM20*, *MYBPC3*, *MYH6*, *MYH7*, and *JUP*). *VCL* at position 75854211 had an allele frequency hit of 0.0000009 which is considered so low and is still viewed as disease causing. Similarly ABCC9 with 0.000041 incidence. PolyPhen 2 shows *VCL* at position 75830737 and 75874563 both to be clinically damaging. *MYBPC3* at position 47356640 is possibly damaging with a score of 0.602, similarly at position 47371330 with a score of 0.907. Also *ABCC9* and *JUP* on position 39925903 reveal to be clinically damaging by PolyPhen 2.

MR 8	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense					
TTN			x	G	C	179472934	HOM	High Freq
MYH6			x	C	A	23853791	HOM	NOT FOUND
TTN	Deletion			TGGG	T	179453362	HOM	High Freq
TTN		x		T	G	179472925	HOM	High Freq
TTN		x		G	C	179472928	HOM	High Freq

Table 20. MR8 Haloplex sequencing results show a novel variant in *MYH6* at position 23853791 of a nonsynonymous nonsense resulting in a stop codon. This is of high importance due to the drastic implication of a stop codon on the protein translated.

MR 9	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame -shift	Non-synonymous Missense	Non-synonymous Nonsense					
NO MUATATION DETECTED								

Table 21. MR9 Haloplex sequencing results reveals no variant in the tested genes.

MR 10	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense					
TTN		x		C	T	179545859	HOM	
TTN		x		C	T	179623758	HOM	
TTN		x		C	T	179629461	HOM	
TTN		x		G	A	179644035	HOM	
TTN		x		T	C	179644855	HOM	
TMEM43		x		A	T	14174427	HET	
TMEM43		x		T	C	14175262	HET	
SCN5A		x		G	A	38603958	HOM	0.00023
SCN5A		x		T	C	38645420	HET	
LDB3		x		C	T	88485923	HET	
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
MYBPC3		x		C	A	47353692	HOM	NOT FOUND
MYBPC3		x		C	T	47371598	HET	
PKP2		x		A	G	33021934	HET	
PKP2		x		T	C	33030802	HET	
JUP		x		T	A	39912145	HOM	0.6592
JUP		x		C	T	39925713	HET	
DSG2		x		A	G	29104714	HET	

Table 22. MR10 Haloplex sequencing results show one interesting variant in *MYBPC3* at position 47353692. PolyPhen 2 shows that this variant is probably clinically damaging.

MR 16	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense					
NEXN		x		G	A	78392446	HOM	0.1846
TNNT2		x		G	C	201333481	HOM	NOT FOUND
TNNT2		x		C	A	201333482	HOM	NOT FOUND
TTN		x		C	T	179545859	HET	
TTN		x		C	T	179623758	HOM	High Freq
TTN		x		C	T	179629461	HOM	High Freq
TTN		x		C	T	179637875	HOM	High Freq
TTN		x		G	A	179644035	HOM	High Freq
TTN		x		T	C	179644855	HET	
TTN		x		G	A	179659912	HET	
SCN5A		x		T	C	38645420	HET	
VCL		x		G	A	75834653	HOM	NOT FOUND
RBM20		x		C	T	112572418	HOM	0.00004
RBM20		x		G	C	112572458	HOM	0.9968
RBM20		x		G	C	112595719	HOM	0.7637
MYBPC3		x		C	T	47360181	HET	
ABCC9		x		C	G	22012554	HOM	0.000008
ABCC9		x		A	T	22012560	HOM	0.000008
MYL2		x		G	T	111351120	HOM	NOT FOUND
MYH6		x		G	T	23853833	HOM	NOT FOUND
MYH6		x		A	G	23861811	HET	
MYH7		x		C	A	23884310	HOM	NOT FOUND
TCAP		x		C	A	37822349	HOM	NOT FOUND
JUP		x		T	A	39912145	HOM	0.6592
TNNI3		x		C	T	55665410	HET	

Table 23. MR16 Haloplex sequencing results show novel variants in *NEXN*, *VCL*, *TTN2*, *MYL2*, *MYH6*, *MYH7*, and *TCAP* at different positions. *RBM20* at position 112572418 along with *ABCC9* at positions 22012554 and 22012560 are also considered important due to their low prevalence and allele frequency. *VCL*, *RBM20, ABCC9* at position 22012554 and 22012560, *MYH6, MYH7,* and *TCAP* show high probability to be clinically damaging through PolyPhen 2.*MYL2* shows a moderate damaging ability with a score of 0.897.

MR 21	Mutation			Ref Allele	Altered Allele	Position	Change	Database (EXAC) Allele Frequency
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense					
TNNT2		x		T	C	201330429	HET	
TNNT2		x		C	G	201333474	HOM	NOT FOUND
TNNT2		x		G	C	201333481	HOM	NOT FOUND
TNNT2		x		C	A	201333482	HOM	NOT FOUND
TMEM43		x		T	C	14175262	HET	
SCN5A		x		A	T	38603955	HOM	NOT FOUND
SCN5A		x		T	A	38603959	HOM	NOT FOUND
TNNC1		x		C	T	52485868	HOM	NOT FOUND
SGCD		x		G	A	155935708	HET	
SGCD		x		G	T	156186364	HET	
RBM20		x		G	T	112581294	HET	
JUP		x		T	A	39912145	HOM	0.6592
DSG2		x		A	G	29104714	HET	

Table 24. MR21 Haloplex sequencing results reveals TTN2 at 3 different positions, SCN5A at 2 different positions, and TNNC1. PolyPhen 2 reveals *TNNC1* to be the damaging variant.

2. SureSelect: Whole Exome Sequencing

As a first approach to analyze the whole exome sequencing, we searched for variant in the 34 genes implicated in cardiomyopathy, then we were interested in filtering the non-synonymous nonsense which results in a stop-gain effect and that may have serious implications on the protein level and consequently the disease state. Results of the 3 of the 7 patients, which revealed interesting results, are presented in the following tables.

EGY-1	Mutation			Ref Allele	Altered Allele	Change
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense			
LARP-6			x	C	A	Het

Table 25. EGY-1 SureSelect sequencing results reveal a potential novel gene non-synonymous non sense in LARP-6 which is proven to be highly expressed in the heart.

MR 37	Mutation			Ref Allele	Altered Allele	Change
Gene	Frame-shift	Non-synonymous Missense	Non-synonymous Nonsense			
LDB3		x		T	C	Hom
AR	x			G	-	Hom

Table 26. MR 37 SureSelect sequencing results reveal LDB3 mutation as a non-synonymous missense which is a known gene to cause cardiomyopathy. Further, androgen receptor (AR) which participates in normal cardiac growth is mutated as frame-shift in our patient.

MR 38	Mutation			Ref Allele	Altered Allele	Change
Gene	Frame-shift	Nonsynonymous Missense	Nonsynonymous Nonsense			
MYH15			x	G	A	Hom

Table 27. One of our patients reveals a mutation in MYH15 as a non-synonymous nonsense mutation where variants in this gene are associated with both non-cardioembolic stroke and coronary heart disease.

CHAPTER IV

DISCUSSION

Cardiomyopathy is a disease in which the heart muscle weakens and becomes enlarged, hampering its ability to efficiently pump blood throughout the body and leading, possibly, to heart failure. There are multiple types of cardiomyopathy, and risk factors include a genetic component. They are classified into several groups according to the morphological and functional phenotype: hypertrophic, dilated, restrictive left ventricular, arrhythmogenic right ventricular dysplasia, and left ventricular hypertrabeculated or non-compaction cardiomyopathy (Richardson 1996, Maron 2006). Thus, cardiomyopathy is now classified as a hereditary disease caused by mutations in one of a panel of culprit genes identified so far. The mutations identified are thought to account to the highest causative agent behind the disease (65-75% of patients) (Maron 2006). Though the genetics behind this complication is well established, making use of the genetic data to manage the disease is still facing many challenges. One of the encountered hurdles is the burden the physicians, genetic counselors, and family members face when dealing with the results of the genetic screening. Additionally, the difficulty of determining the treatment or follow-up regimen that should be adopted in order to prevent SD of patients who are found to be genotype positive for the discovered mutations and yet phenotype negative and thus who have no clinical disease.

In the Europeans, African-Americans, and Asian populations, genetic studies have been conducted extensively focusing on genetic susceptibility for cardiomyopathies.

Plakophilin-2 c.419C>T is associated recently with risk of heart failure and arrhythmias in the European population (Christensen 2015). Similarly, a common variant rs7597774 in *β-adducin* gene (*ADD2)* is associated with DCM in Chinese Han population (Chen 2015). This is a new gene to be reported relating to cardiomyopathies. Moreover, four mutations (1 novel) are identified in Indian HCM cases, namely, Pro82Ser, Arg98Gln, Arg141Gln and Arg162Gln in *Troponin I* protein (Ramachandran 2013). Moreover, a homozygous truncation mutation c.1660C>T (p.Q554X) in *desmocollin-2* (*DSC2)* leads to a cardiac-restricted phenotype of an early onset biventricular arrhythmogenic cardiomyopathy (Gerull 2013).

Middle Eastern populations, however, have been only rarely studied. We hypothesized that existing and/or novel mutations in the panel of genes are responsible for the majority of the cardiomyopathies that are frequently diagnosed in patients admitted to the AUBMC, Lebanon. Under the umbrella of the Dubai Harvard Foundation for Medical Research grant, one of our lab members has also screened for mutations in a familial case of HCM with severe phenotype and multiple cases of SD at early age using the NGS Platform Technology at Harvard Medical School. Results identified an R21C mutation in the *Troponin I* gene (*TNNI*) to be the underlying cause of the disease (Nemer 2013, unpublished data). Only one previous report showed this same mutation to be the cause of HCM in a family of Syrian origin described by Seidmans at Harvard 25 years ago . While compiling the data, it was noted that one of the parents of the Syrian family in question is from Lebanon and is the one transmitting the mutation (Figure 13).

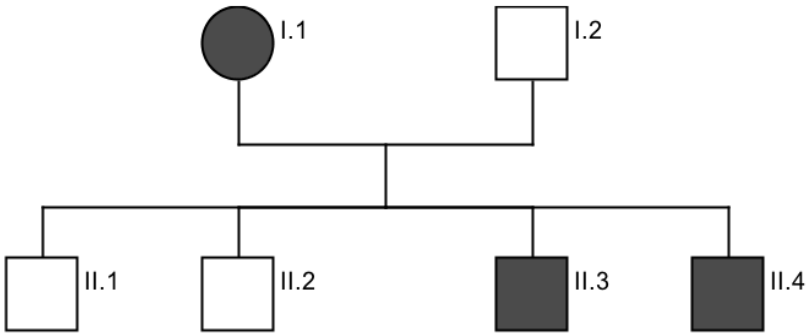


Figure 13. Representation of the family affected with the R21C mutation in Troponin I.

We focused in our current results, using Next Generation Haloplex Targeted sequencing, on homozygous mutations since none of our 19 consented patients had a positive parent cardiomyopathy history. Our results show that there are many homozygous genes from the cardiomyopathy panel that are mutated. These are in the form of non-synonymous missense in 2 mutations in *NEXN* (K135E) (G181R), 6 mutations in *SCN5A* (L699R) (A1878V) (T38N) (A1240S) (L1215Q) and (T1250S), 3 mutations in *DSP* (V1968D) (I305F) and (E1181D), 3 mutations in *TGFβ3* (C409R) (K407R) (K407Q) and (E169D), 2 mutations in *MYH7* gene (S1491C) and (R1818L), 2 mutations in *LMNA* (P291T) and (A376S), 1 mutation in *DES* (W295C), 3 mutations in *VCL* (P291T) (R132L) and (R1055Q), 3 mutations in *MYH6* (E1744D) (L772I) and (M547I), 2 mutations in *RBM20* (P1059T) and (E1223Q), 2 mutations in *MYBPC3* (S217G) and (G1249C), 2 mutations in *JUP* (T79P) and (T78A), 3 mutations in *TNNT2* (A130G) (A130S) and (Q132H), 1 mutation in *MYl2* (P95T), 1 mutation in TCAP (A164E), and finally TNNC1

mutation (G70D). We noticed a high occurrence of certain genes in the study population we had suggesting that these are not disease causing. These are: *RBM20* mutation at position112572458 and position 112595719 which were found in 10 of our patients, conferring the change in amino acids (W768S) and (E1223Q) respectively. Also, the high prevalence of *JUP* at position 3991214514 (M697L) and *TTN* at position 1796440035 (S1249L) suggesting that they are normally present and not disease causing. Exac analysis also showed high allele frequency (a number close to 1) in the normal population of these gene mutations confirming that these variants are non-disease causing. Some patients did not show any significant mutation in the panel of cardiomyopathy genes tested. Those patients are excellent candidates for whole exome sequencing which might be more informative in their case for the possibility of finding novel genes causing the cardiomyopathy seen rather than mutations in the genes that are already discovered.

As well known, the cardiac muscle is a striated involuntary muscle. The striations are due to the basic unit of the muscle: the sarcomere. A sarcomere is defined as the segment between two neighboring Z-lines (or Z-discs). They are composed of long, fibrous proteins (myosin and actin) that slide past each other when the muscles contract and relax. The cardiac contraction and mechanical function are generated by the sarcomere. When the muscle is activated by the membrane action potential, which changes the intracellular free calcium concentration, the myosin heads interact with the actin binding sites. This interaction creates cross – bridges which are the motor units of the sarcomere. Those bridges are regulated on the thin filament by the complex of tropomyosin and troponin with the three subunits: troponin I, troponin T, and troponin C. In addition to the importance of the sarcomeres in the cardiac muscle, there are the desmosomes which localized to

intercalated discs and mechanically couple cardiac cells to function in a coordinated syncytial structure. Our results showed several novel mutations mainly in sarcomeric genes: *NEXN*, a Z-disc gene which encodes a filamentous actin-binding protein nexillin: it has a unique role in protecting Z-disks from mechanical trauma. Mutations in nexilin have been proven to destabilize cardiac Z-disks (Hassel 2009). *DES,* which encodes desmin protein, a muscle-specific, type III intermediate filaments that integrates the sarcolemma, Z disk and nuclear membrane in sarcomeres and regulates sarcomere architecture. *MYH7* encodes β-myosin heavy chain that is part of a larger protein called type II myosin that is integral to sarcomeres. The latter generates the mechanical force that is needed for muscles to contract. *MHY6* encodes myosin heavy chain isoform, predominantly found in the atria and also it is major part of contraction. *MYBPC3* gene which provides instructions for making the cardiac myosin binding protein C (cardiac MyBP-C) that is also associated to the sarcomeres by attaching to thick filaments and keeping them from being broken down. *TNNT2* encodes troponin T and *TNNC1* encodes troponin C and as mentioned earlier those proteins are part of a big complex that regulates contraction. *TCAP* encodes telethonin protein which is expressed in cardiac muscles at Z-discs and functions to regulate sarcomere assembly. *DSP* encodes desmoplakin, *JUP* encodes plakoglobin, *VCL* encodes vencullin which are all critical components of desmosome structures in cardiac muscle.

PolyPhen 2 testing showed novel clinically damaging mutations to be in: *TGβ3* (C409R) (K407R) (K407Q) and (E169D), *DSP* (E1181D) *MYH7* (R1818L), *DES* (W295C), *VCL* (A259T), (R132L) and (R1055Q), *ABCC9* (R661C), (V822E) and (R824P), *RBM20* (R755C), *MYBPC3* (S217G) and (G1249C), *MYH6* (L772I) and (L1795M), *JUP* (T79P), *MYl2* (P95T), TCAP (A164E), and finally TNNC1 (G70D).

Among our results also there were non-synonymous nonsense mutations that resulted in stop codons and were of clinical significance. These are *TTN* (S14991*), *MYH6* (G1809*), and *LDB3* (C444*) seen in the family of an RCM patient. One novel mutation in *MYH7* resulted in exon splicing and we suggest having serious implications on the clinical manifestation of a patient. *TTN* gene encodes titin and LDB3 gives instructions for making a protein called LIM binding domain 3, both are essential components of the sarcomere and, thus, their mutation would expectedly affect contraction and cause disease.

For each patient sequenced using the SureSelect platform, more than 80,000 variants were validated and analyzed by SureCall software. We grouped them according to their effect on protein as the HaloPlex platform. The first patient whole exomed sequenced reveals a stop codon mutation in *La-related protein 6* or *La ribonucleoprotein domain family member 6* (*LARP6).* We speculate that this gene might be novel causing cardiomyopathies since RT-PCR of mouse tissues shoed highest expression of *LARP6* in brain and strong expression in the heart (Valavanis 2007). In another patient, a non-synonymous missense mutation is noted in *LDB3* which is known to cause cardiomyopathy along with a frame-shift mutation in *androgen receptor (AR)* Echo of *AR* knockout mice demonstrated impaired concentric hypertrophic response and left ventricular dysfunction. Angiotensin II stimulation showed cardiac fibrosis in the knockouts compared to wild type. So androgen-androgen receptor system participates in normal cardiac growth and modulates cardiac adaptive hypertrophy and fibrosis during hypertrophic stress (Ikeda 2005). In addition, low number of CAG repeats in the *AR* gene implies a greater chance for low levels of HDL cholesterol and reduced endothelial response to ischemia which are important risk factors for coronary heart disease (Zitzmann 2001). Our patients to re-

emphasize had only idiopathic cardiomyopathy and not any related heart disease. So any drastic mutation (like non-synonymous missense or frame-shift) in a gene related to the heart will be implicated in heart disorder or function impairment. Since our patients did not have any heart related disease except the cardiomyopathy, then we highly suspect that the above mentioned genes are novel genes to cause this disease.

Some of the limitations were that we focused only on homozygous mutations but the combination of compound heterozygous mutations in different genes should be also studied. We should take into account De Novo mutations and copy number variants and this requires genotyping the parents.

Thus, future work is to screen family members to confirm phenotype-genotype associations, confirm novel mutations by Sanger sequencing and consequently link the confirmed mutation to a specific cardiomyopathy type of the patients. HaloPlex patients that did not show any significant mutation are excellent candidates for SureSelect sequencing aiming to find a novel gene causing this cardiomyopathy. Results should be validated against a control group to confirm the nonexistence of the genes that we suspect to cause the cardiomyopathy, and finally perform in vivo studies for the suspected novel genes of SureSelect. Those genetic studies will aid in establishing an efficient genetic counseling coupled with a broader understanding of the genotype/phenotype correlations. There is a necessity of finding the functional mechanisms causing the cardiomyopathy disease and the discrepancy in the phenotypes should be studied in order to put effectively a treatment regimen to handle all types of patients regardless if there is an inherited mutation or not, since 25-35% of cardiomyopathy patients have no pathogenic mutations asserting that there are novel mutations to be discovered.

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