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DIFFERENT METHODS TO MODEL CARDIAC ARRHYTHMOGENIC DISEASES: FROM TRANSFECTED CELLS TO CARDIOMYOCYTES DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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INTRODUCTION

CARDIOMYOPATHIES

General features and classifications

Cardiomyopathies are a heterogeneous group of diseases of the heart muscle that involve directly the contractile tissue (not indirectly like heart failure and hypertension), affecting the capability of the heart to act as a pump. They could arise from a diverse array of conditions (infections, ischemia or toxin exposure) that damage the heart and other organs, resulting in the impairment of the cardiac function the observation of a familial heritance in the disease occurrence led doctors to investigate the role of genetic mutations in the onset of the pathology; in fact, genetic mutations are actually considered as the most relevant causes of cardiomyopathy, which were considered idiopathic for a long time. Cardiomyopathies, with their heterogeneity of causes, severities and progression, have in common some manifestations, such as the occurrence of arrhythmia, heart failure and sudden death. It has been very difficult to characterize these diseases and classify them in a useful way, to better diagnose and treat them.

The concept of isolated, non-ischemic disease of the myocardium dates back to 1899 when Fiedler described a series of fatal cases in young adults with cardiomegaly and heart failure. A classification serves to bridge the gap between ignorance and knowledge[1]. Maybe, is because this gap is large still today, that we had to wait until 1961 to have the first classification of these “rare” diseases, of the muscular cells of the heart, called cardiomyopathies. Until 1961, researchers and doctors referred of an “uncommon myocardial disease” defined as “heart muscle disease of unknown cause” to differentiate it from those of known causes. When Gordon, Hollman and Bishop give us the first definition of cardiomyopathy they wrote: “cardiomyopathy: an acute, subacute or chronic disorder of heart muscle of unknown or obscure aetiology, often with associated endocardial or sometimes with pericardial involvement but not atherosclerotic in origin"[2]; it was the 1964 and then they simplified to "a disorder of cardiac muscle of unknown cause”. From this first step in defining and characterizing the disease, it is clear how difficult it was to understand the underlying mechanisms and, thanks to its great variability in phenotypes, how difficult it was to identify, diagnose and treat. In the first 80’s, a task force of the World Health Organization, chaired by John Goodwin presented the first classification of the cardiomyopathies based on structural features and hemodynamic phenotype; they divided cardiomyopathies in Dilated (DCM), Hypertrophic (HCM) and Restrictive (RCM)[3]. Subsequent

[1] *Goodwin JF. The frontiers of cardiomyopathy 1986*
[2] *Goodwin JF. Cardiac function in primary myocardial disorders. Br MedJ 1964*
[3] *Report of the WHO/ISFC task force on the definition and classification of cardiomyopathies. Br Heart J. 1980*

iterations of the classification updated the diagnostic criteria in relation to evolving diagnostic technology (eg, angiography, M mode, and then 2-dimensional echocardiography). Then, in 1990, the discovery of a mutation in the β-myosin heavy chain gene, in 20 members of a French/Canadian family affected by HCM, led to discover that cardiomyopathies could be linked to genetic alterations of sarcomere proteins, so the disease was no longer idiopathic[4][5]. After this important discovery, it was proposed that these disorders could be classified according to the molecular genetic defect: sarcomeric cardiomyopathy, cell junction cardiomyopathy, ion channel cardiomyopathy, cytoskeletal cardiomyopathy,[6] *etc.* In 2006, a new classification was proposed by the American Heart Association (Fig.1), which reflected the evolving genetic knowledge about the cardiomyopathies; those were considered to be "primary" when the disease was solely or predominantly involving the heart muscle and "secondary" when myocardial involvement was associated with a multisystemic, generalized disorder. Primary cardiomyopathies (CMs) could be genetic, acquired or mixed[7]. In this classification, also ion channel disorders are included as genetic cardiomyopathies; indeed, ion channelopathies shows a grossly normal heart, but are diseases predisposing to potentially lethal ventricular tachyarrhythmias and characterized by genetic mutations in ion channel proteins, leading to dysfunction in sodium, potassium, calcium, and other ion channels, thus compromising the capability of the heart muscle to contract and act as pump. Like the other type of genetic cardiac diseases, even if they do not change the anatomy of the heart, channelopathies affect the heart function damaging the contractile properties of cardiomyocytes; this led to classify them as cardiomyopathy[8]. Disorder in ion channel function, even in a heart with normal systolic and diastolic function, leads to an abnormal architecture of the heart tissue and to electrical dysfunction that are, indeed, disorders of the cardiomyocytes. That's why the American heart association decided in 2006 to consider them as genetic primary cardiomyopathies. Also, genes encoding specific proteins may cause very different phenotypes. The fact that mutations in sarcomeric genes are associated with HCM and restrictive cardiomyopathy, whereas dilated cardiomyopathy may be caused by genes encoding not only sarcomeres' proteins, but also cytoskeletal and even ion channel and gap junction proteins, provides the challenge of discovering how these mutations are translated into such different

[4] *Olson TM "Actin mutations in dilated cardiomyopathy, a heritable form of heart failure". Science. 1998;*

[5] *Geisterfer-Lowrance AA "A molecular basis for familial hypertrophic cardiomyopathy: a beta cardiac myosin heavy chain gene missense mutation." Cell. 1990*

[6] *Thiene G, Corrado D, Basso C. "Cardiomyopathies: is it time for a molecular classification?" Eur Heart J. 2004*

[7] *William J. McKenna. "Classification, Epidemiology, and Global Burden of Cardiomyopathies" Circ. Res. 2017*

[8] *Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, Moss AJ, Seidman CE, Young JB; American Heart Association; Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; Council on Epidemiology and Prevention. Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. Circulation. 2006*

phenotypes. Of note, the European Society of Cardiology classification (Fig.1) avoids the use of "primary" and "secondary" cardiomyopathy, because these distinctions may not be readily discernible. Furthermore, some diseases do not have a uniformly static expression and may evolve, as a consequence of remodelling, from one category to another during their natural clinical course; so etiologic classifications, has limited value for clinicians because management strategies are dynamic and inevitably evolve during the diseases[9].

Primary cardiomyopathies could be genetic, mixed or acquired; acquired cardiomyopathies should not be confused with secondary cardiomyopathies. Indeed, while secondary cardiomyopathies are a consequence of a systemic diseases, acquired CMs are caused by non-genetic factors that leads directly to myocardium dysfunction and cardiac complications. Anyway, primary or secondary cardiomyopathies have in common some hallmarks manifestations that could be mild or severe, in a range that goes from microscopic alteration of cardiac myocytes physiology to arrhythmias and heart failure[9]. It is crucial that primary care physicians recognize the evidence of a cardiomyopathy and start to investigate it, to identify the right phenotype. Usually, cardiomyopathies present with similar symptoms, even if they involve a variety of aetiologies; those symptoms include peripheral oedema, fatigue, syncope or pre-syncope and symptomatic cardiac ischemia. Diagnostic measurements start with electrocardiography and echocardiography, which may help to identify the phenotype. It is important to understand the origin of the pathology to better treat symptoms; cardiomyopathies could have different underlying causes, each one triggering a specific pathway that led, in the end, to similar alterations of the cardiac function. Different aetiologies include defect in force generation or transmission, energy deficit, abnormal calcium homeostasis or hypersensitivity of myofibrils to calcium. These different mechanisms could be treated specifically[10] only if they were known, in over 50% of cases specific causes of disease are not found; that's why cardiomyopathies are often classified as idiopathic. However, with a better investigation of the causes, the treatment will also be more specific and hospitalization may not be necessary. Nowadays, many cases remain undefined and treatment is usually not specific. In the last years, also genetic testing emerged as an important tool to identify the cause of cardiomyopathies. Autosomal dominant, autosomal recessive or x-linked patterns of inheritance have been identified and suddenly researchers started to identify the genes related to the different types of cardiomyopathies.

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[9] *Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, Moss AJ, Seidman CE, Young JB; American Heart Association; Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; Council on Epidemiology and Prevention. Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention.* ***Circulation****. 2006*

[10] *Dadson K, Hauck L, Billia F. Molecular mechanisms in cardiomyopathy. Clin Sci (Lond). 2017*

As mentioned above, in the 1990 the first gene related to a cardiomyopathy was identified: It was the beta-myosin heavy chain gene, responsible for genetic cardiac hypertrophic cardiomyopathy [11]. Since then, much progress has been made in elucidating gene related defects in all types of cardiomyopathies (hypertrophic, dilated, restrictive and channelopathies); however, identifying the genetic cause, not always helps to explain the mechanisms behind the pathologic manifestations; for instance, the same genes involved in hypertrophic cardiomyopathies are involved also in dilated cardiomyopathy (DCM), without no evidence of why some mutations cause HCM, while other mutations on the same genes cause DCM; on the other side, different pathways and mutations in different genes could lead to similar phenotypes. Recent studies investigate the similarities among the profiles of different genetic mutations responsible for arrhythmogenic cardiomyopathies like Brugada syndrome, long QT 3 syndrome and HCM; in the end, even if mutations causing the disease are different, all involve the failure of the cardiac sodium channels, of the kinetics of calcium transients and alterations of CAMKII activity. These mechanisms lead to diastolic dysfunction, arrhythmias and heart failure, even before the anatomic remodelling occur. Starting from this new point of view, is clear how difficult it could be to find the right pharmacological or surgical treatment for patients affected by cardiomyopathies, if we only look at the genetic cause or at the anatomic remodelling, without an overall view on the pathology, comprehensive of the symptoms, the genetic background, the family and patient history of adverse events. The phenotype also depends on the individual response to the causing event, and carrying out the appropriate pharmacological treatment could be essential to avoid hospitalization, heart transplantation or implantable cardiac devices (ICD); that's why researchers are increasingly oriented to personalized therapies.[12] The best way to reach this purpose is of course to have an experimental model of the disease, such as animals carrying the same mutations found in patients, or an expression system. However, if we really want to observe the patient specific response to pharmacological treatments, it is better to have a system where all the proteins are expressed and the regulatory pathways are activated, like in the "real" human heart tissue; for this reason, the use of human induce pluripotent stem cell derived cardiomyocytes (hiPSC-CM) represents the best option for studies of drug discovering and toxicology for these pathologies. [13] The new technology of hiPSC-CM allows us to obtain cardiomyocytes directly from patients, so those cells express the patient's genetic pool and enable us to study in vitro the patient-specific pathology.[14]

[11] *Geisterfer-Lowrance AA, Kass S, Tanigawa G, et al. A molecular basis for familial hypertrophic cardiomyopathy: a beta cardiac myosin heavy chain gene missense mutation. Cell. 1990*
[12] *Fatkin D, Graham RM. Molecular mechanisms of inherited cardiomyopathies. Physiol Rev. 2002*
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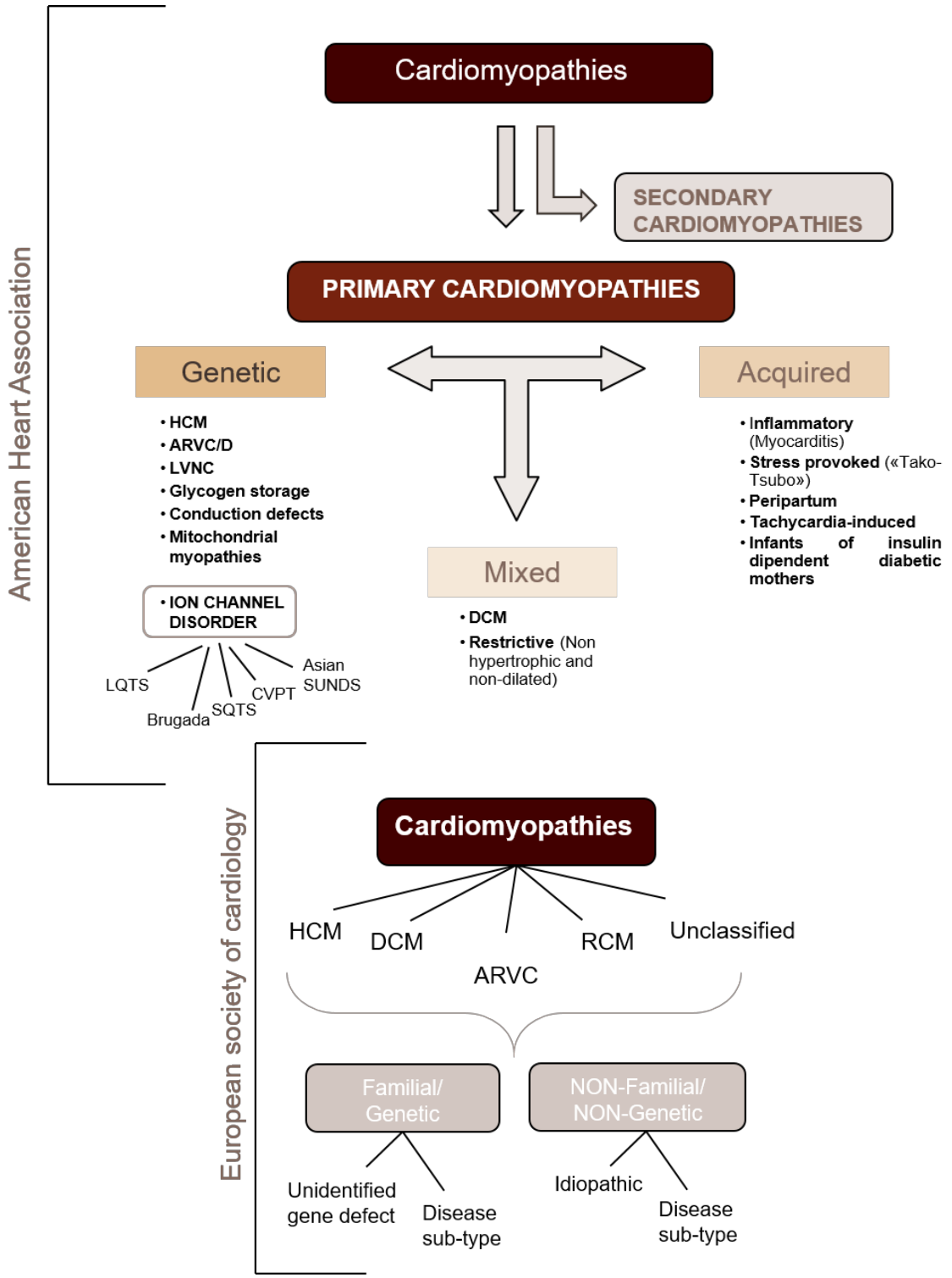

Figure 1: Classification of cardiomyopathies

In the top box: The American Heart Association classification of cardiomyopathies, that makes a distinction between primary and secondary cardiomyopathies on the base of the genetic involvement, considering cardiomyopathies to be "primary" when the disease predominantly involves the heart muscle and "secondary" when is associated with a multi systemic, generalized disorder. The European society of cardiology focuses the classification on the structural and hemodynamic features of the disease and it takes into account the remodelling promoted by

cardiomyopathy that makes it hard to classified and treat specifically, thus avoiding the primary and secondary classification.

Epidemiology and main phenotypes

Inherited cardiomyopathies are a major cause of heart disease in all age groups, often with an onset in adolescence or early adult life[15]. Reliable epidemiology of the cardiomyopathies is available predominantly from developed countries, where accurate prevalence data, that rely on application of established diagnostic evaluations and criteria, are gathered. Among the most common phenotypes of cardiomyopathy, we can identify HCM, DCM and channelopathies like LQT syndrome or Brugada syndrome. HCM is a common inherited cardiomyopathy and it represents a frequent cause of sudden cardiac death in the young. The identification of an increase in the wall thickness of the left ventricle (over 15 mm), not explained by the presence of secondary hemodynamic causes, is necessary for the diagnosis of HCM. It has a prevalence of 1:250 to 500 in adults; the prevalence seems to be similar in all races and ethnicities[16],[17],[18]. HCM is the most common genetic cardiac disease and an important cause of sudden death for cardiac cause in people under 50 years of age and in athletes. The current prevalence of HCM is based on echocardiographic studies, so it is easy to think that a large part of affected but asymptomatic patients remain unknown. Many of those asymptomatic patients do not recognize any cardiovascular abnormalities, except for the onset of lethal arrhythmias that are often the first symptom. Asymptomatic subjects are at high risk for lethal cardiac events and they miss the opportunity to be treated properly[19].

DCM has an incidence of approximately 1:2500 in the general population and males are three times more susceptible than females; robust data on the epidemiology of DCM are lacking. It is one of the most common indications for heart transplantation and the echocardiographic test is necessary for diagnosis and it is actually found more frequently in young adults[20] . Familial DCM is the 30-50% of the total DCM population, it usually has an autosomal dominant inheritance, but it could also be transmitted with a x-linked pathway[21].

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[15] *Redwood C. Inherited Cardiomyopathies. N Engl J Med 2011*

[16] *Olivotto I, Cecchi F. "The epidemiologic evolution and present perception of hypertrophic cardiomyopathy." Ital Heart J. 2003*

[17] *Maurizi N, Ammirati E, Coppini R, Morrone A, Olivotto I. Clinical and Molecular Aspects of Cardiomyopathies: Emerging Therapies and Clinical Trials. Heart Fail Clin. 2018*

[18] *Maron BJ, Rowin EJ, Casey SA, et al. "How hypertrophic cardiomyopathy became a contemporary treatable genetic disease with low mortality: shaped by 50 years of clinical research and practice." JAMA Cardiol 2016*

[19] *Maron MS, Hellawell JL, Lucove JC, Farzaneh-Far R, Olivotto I. Occurrence of Clinically Diagnosed Hypertrophic Cardiomyopathy in the United States. Am J Cardiol. 2016*

[20] *Kirk R, Naftel D, Hoffman TM, et al. Outcome of pediatric patients with dilated cardiomyopathy listed for transplant: a multi-institutional study. J Heart Lung Transplant. 2009*

[21] *Elliott P, Andersson B, Arbustini E, et al. Classification of the cardiomyopathies: a position statement from the European Society Of Cardiology Working Group on Myocardial and Pericardial Diseases. Eur Heart J. 2008*

Channelopathies involve many different alterations in the cardiac channels, each causing a different phenotype; taken together, channelopathies are responsible for the 40% of sudden death for natural cause in <35 years of age, as they render the affected, susceptible to different cardiac arrhythmias. Among channelopathies, Long QT syndrome (LQTs), has a prevalence of 1:2000; it has been found also in neonates and data suggest that this is similar for all ages and races but female seems to be more susceptible than male subjects[22] [23]. Brugada syndrome (BrS) affects more males than females and has a prevalence of 1:10000, higher in East Asia[24]. Those two are the most common channelopathies, while short QT syndrome (SQTs) is very rare and catecholaminergic polymorphic ventricular tachycardia (CPVT) occurs in less than 1:10000. Diagnosis of channelopathies required the identification of specific abnormalities in the electrocardiogram (ECG): indeed, each defect in channel function reflects on cardiac electrocardiogram; for instance, in LQT syndrome, the QT segment is prolonged over 500 msec, while in Brugada Syndrome there is an elevation of the ST segment, followed by ST depression. Those features could be hidden sometimes, so uncovering them with drugs is essential to make the diagnosis of channelopathy.[25]

Overall, cardiomyopathies are a primary problem. A cardiomyopathy was present in more than half of patients <35 years dying suddenly or requiring cardiac transplantation in a study on patients of an Italian hospital cardiac division[26] . Cardiomyopathy represent a high risk for cardiac arrhythmias and sudden death, and patients need to be treated properly to avoid those effects and diagnosing the correct phenotype is essential for selection of the right pharmacological treatment[27].

Hypertrophic Cardiomyopathy: HCM is a common genetic disorder characterized by left ventricle hypertrophy not explained by abnormal loading conditions[28]. HCM was termed a "disease of the sarcomere" because the first mutations causing this disease were found to encode for components of sarcomeres, the contractile apparatus of the heart muscle. Nowadays, more than 1400 pathogenic variants in over 20 genes encoding thick and thin filament proteins of the

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[23] *Skinner JR, Winbo A, Abrams D, Vohra J, Wilde AA. "Channelopathies That Lead to Sudden Cardiac Death: Clinical and Genetic Aspects." Heart Lung Circ. 2019*

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[26] *Thiene G. "Sudden cardiac death and cardiovascular pathology: from anatomic theater to double helix". **Am J Cardiol**. 2014*

27 Milman A, Andorin A, Gourraud JB, et al. Age of First Arrhythmic Event in Brugada Syndrome: Data From the SABRUS (Survey on Arrhythmic Events in Brugada Syndrome) in 678 Patients. Circ Arrhythm Electrophysiol. 2017

[28] *Maurizi. N, Enrico A., Coppini R, Morrone A., Olivotto I. "Clinical and Molecular Aspects of Cardiomyopathies: Emerging Therapies and Clinical Trials" Heart Failure Clin 2018*

sarcomere or the components of the adjacent Z-discs have been discovered as causes of HCM; most variants are transmitted in an autosomal dominant pattern. Among patients with sarcomeric HCM, about 70% show pathogenic variants in either the genes that encode β-myosin heavy chain (MYH7) or myosin binding protein C (MYBPC3). Conversely, troponin T (TNNT2), cardiac troponin I (TNNI3) and α tropomyosin (TPMI), account for about 5% of cases each[29] [30]. Mutations causing HCM are usually gain of function, producing over activation of the contraction machinery, and metabolic changes, leading to energy depletion and triggering fibrosis. Increased thin-filament calcium sensitivity and changes in signalling pathways mediated by cMyBP-C, related to cardiac hypertrophy, are likely to contribute to the diastolic dysfunction, a hallmark of HCM. The elevated sarcoplasmic calcium concentration during diastole also promote hypertrophic signalling (e.g., by means of calcineurin–nuclear factor of activated T cells (NFAT) and calcium-calmodulin–dependent protein kinase II (CaMKII))[31] and may also confer a predisposition to arrhythmias[32]. Sarcomeric mutations could lead to a hypertrophic profile by, at least, two mechanisms: (i) increasing calcium sensitivity by affecting regulatory proteins of the thin filament; (ii) increasing the ATP needed by myosin ATPase[33]. This could steal ATP from cytosol and reduce the activity of other ATP-consuming processes such as ion pumps. We should not forget that also SERCA is ATP-dependent and the depletion could reduce the re-uptake of calcium in the sarcoplasmic reticulum (SR) during diastole and increase intracellular calcium concentrations; the higher calcium sensitivity slows down relaxation and makes diastole less efficient.

HCM is associated with complex electrophysiological remodelling at cellular level; it is characterized by a very complex pathophysiological background, reflecting into heterogeneous clinical manifestations and natural history[34]. Besides ATP depletion, altered Ca^{2+} handling is also an important feature of the disease. The altered Ca^{2+} fluxes in HCM myocytes are the result of several concurrent alterations: increased amplitude and slower inactivation kinetics of L-Type Ca^{2+} current, reduced expression of SERCA and reduced SERCA/phospholamban ratio, loss or disorganization of t-tubules, increase leakage of Ca^{2+} from the SR, and abnormal function of the Na^{+}/ Ca^{2+} exchanger (NCX). The latter is the consequence of the increased intracellular concentration of Na^{+}; even the late sodium current (I_{NaL}) was markedly increased in isolated

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[30] *Bos JM, Towbin JA, Ackerman MJ. "Diagnostic, prognostic, and therapeutic implications of genetic testing for hypertrophic cardiomyopathy." Journal of the American College of Cardiology 2009*

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[34] *Spoladore R, Maron MS, D'Amato R, Camici PG, Olivotto I. Pharmacological treatment options for hypertrophic cardiomyopathy: high time for evidence. Eur Heart J. 2012*

human HCM cardiomyocytes, causing sodium overload and the reverse activation of NCX; this mechanism leads to an increased total Ca^{2+} entry during the plateau of the AP, contributing to the formation of a pro-arrhythmic substrate[35]. All the single different pathways and mutations responsible for HCM led to many different phenotypes, from moderate, to serious (with a different likelihood of arrhythmias and sudden cardiac death). To prevent these outcomes, the best option would be a personalized treatment, but patients with HCM are currently treated with different

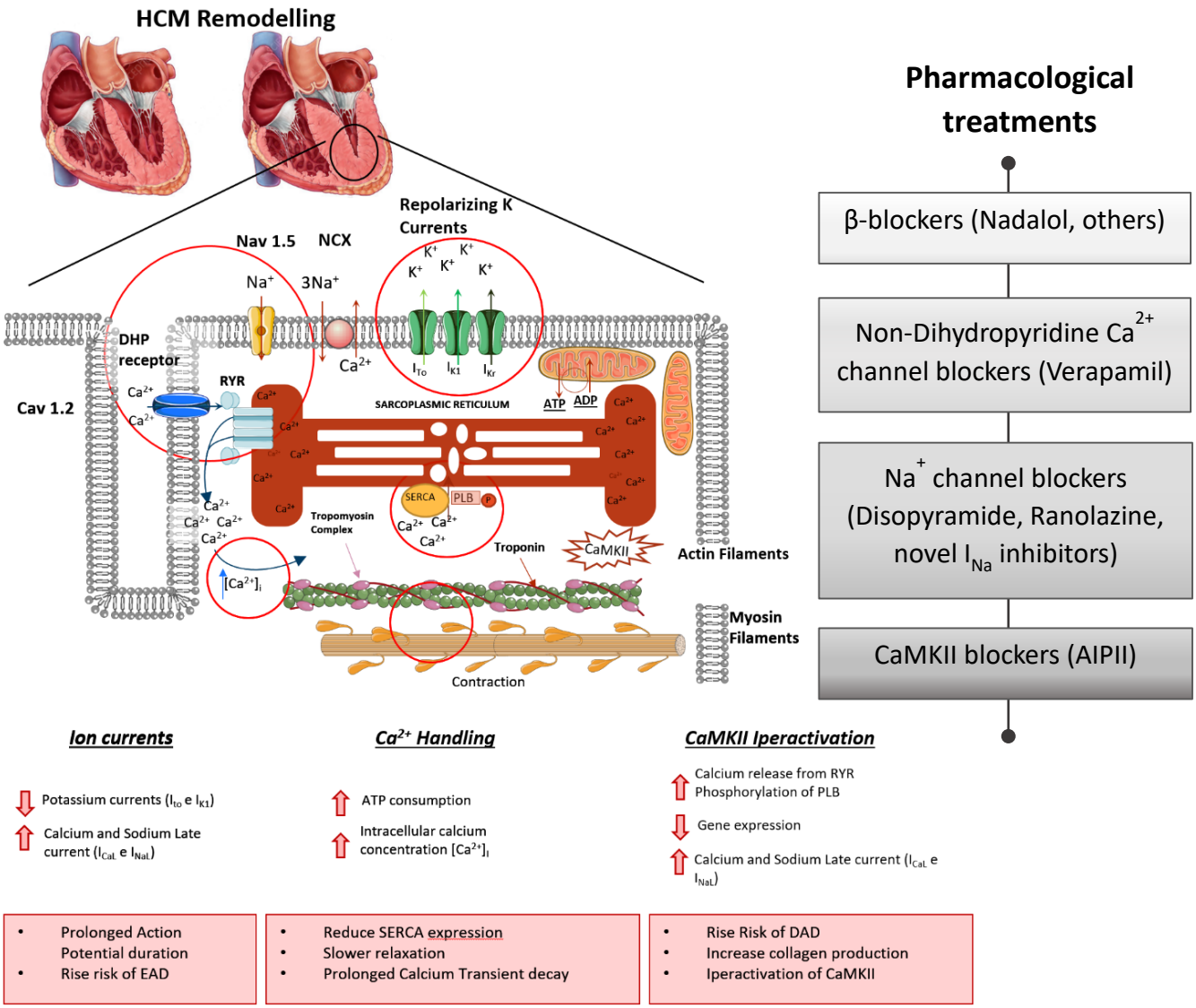

Figure 2: HCM remodelling, impaired mechanisms and pharmacological treatment

In the picture is possible to observe on top the anatomical changes caused by hypertrophic cardiomyopathy, the typical thickening of the interventricular septum and then all the impaired mechanisms; the increase of intracellular calcium and sodium concentration associated with the activation of the reverse mode of NCX, the subsequent ipercontractility of the myofilaments associated with an increase ATP consumption, the iperactivation of CaMKII and the consequent

[35] *Coppini R, Ferrantini C, Mugelli A, Poggesi C, Cerbai E. Altered Ca2+ and Na+ Homeostasis in Human Hypertrophic Cardiomyopathy: Implications for Arrhythmogenesis. Front Physiol. 2018*

reduction of the repolarizing currents (the downstream targets of CaMKII are ryanodine receptors, Ca^{2+} , K^{+} and Na^{+} channel). All is related to an altered gene expression responsible for the reduction of SERCA expression responsible for calcium reuptake and of the potassium channel responsible for cell repolarization. On the right panel the principal pharmacological treatment for HCM.

drugs based on their symptoms. β-Blockers, that represent the mainstay of therapy, have proved effective in patients with angina or dyspnoea on effort, particularly when associated with left ventricular out-flow tract (LVOT) obstruction, and are often employed to reduce the prevalence of non-sustained ventricular arrhythmias[36]. Non-dihydropyridine calcium-channel blockers such as verapamil and diltiazem, have been successfully employed in symptomatic patients with non-obstructive HCM. Conversely, HCM guidelines suggest caution in using calcium channel blockers in patients with significant LVOT obstruction, due to their potentially adverse haemodynamic effects[37] [38] *;* **Disopyramide**, a class I A antiarrhythmic drug, is used to attenuate the pressure gradients and improve symptoms in patients with LVOT obstruction, generally in association with β-blockers[39] . **Amiodarone**, widely used treatment for atrial fibrillation, seems to be highly protective in HCM, with regard to potentially malignant ventricular arrhythmias[40] [41]. Recent studies have also tested some sodium current blockers like **Ranolazine** but their effects seem to be mutation specific[42] [43]. A novel agent, the small molecule **Mavacamten**, an allosteric myosin inhibitor (MYK-461; Myokardia, San Francisco, CA, USA), has recently been developed to restore the physiologic contractile and energetic balance in HCM hearts by decreasing adenosine triphosphatase activity of the cardiac myosin heavy chain[44]

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[36] *Maron BJ, McKenna WJ, Danielson GK, et al. American College of Cardiology/European Society of Cardiology clinical expert consensus document on hypertrophic cardiomyopathy. A report of the American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents and the European Society of Cardiology Committee for Practice Guidelines. J Am Coll Cardiol. 2003*

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[41] *Cecchi F, Olivotto I, Montereggi A, Squillatini G, Dolara A, Maron BJ. Prognostic value of non-sustained ventricular tachycardia and the potential role of amiodarone treatment in hypertrophic cardiomyopathy: assessment in an unselected non-referral-based patient population. Heart. 1998*

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[44] *Green EM, Wakimoto H, Anderson RL, et al. A small-molecule inhibitor of sarcomere contractility suppresses hypertrophic cardiomyopathy in mice. Science. 2016;*

Dilated Cardiomyopathy: DCM is a disease of the myocardium characterized by dilatation of the left ventricle, associated with systolic dysfunction, myocyte death and myocardial fibrosis. It is the second most common cardiomyopathy worldwide and has many causes[45]. Although many patients could be asymptomatic life long, age-dependent penetrance is commonly observed in DCM as in many other cardiac genetic diseases. DCM usually arise in individuals aged 30–60 years, but can also be observed in neonates, infants, children, and adolescents. Dilated cardiomyopathy can rapidly lead to heart failure and life-threating arrhythmias[46]. While several causes of DCM pathogenesis have been identified and genetic mutations are the most common, also inflammation and autoimmunity play an important role and even external factors like the presence of infections or chemical and toxin exposure could trigger DCM. As previews said, gene mutations are the most common causes of DCM, accounting for > 30% of cases; it is mostly inherited in an autosomal dominant fashion with characteristic age-dependent penetrance and variable clinical expression. The genes responsible for the pathogenesis code for a large variety of proteins involved in different subcellular compartments and pathways including also RNA splicing machinery and calcium handling (like in HCM). In DCM, mutations in the genes encoding contractile proteins of cardiomyocytes result in functional changes that are the opposite of the changes caused by mutations in the same contractile genes that cause hypertrophic cardiomyopathy[47]. The molecular mechanisms that determine whether a dilated or hypertrophic phenotype develops in response to a mutation have not been clearly elucidated[48] .

Causative genes seem to encode two major subgroups of proteins: the cytoskeletal and/or sarcomeric proteins. Among the sarcomeric genes affected by mutations causing DCM, recent studies have identified Titin (*TNT*), Myosin heavy chain (*MYH7*), cardiac troponin T (*TNNT2*) and alpha tropomyosin (*TPM1*)[49]. The cytoskeletal proteins involved in DCM development and progression, include dystrophin, desmin, lamin A/C, δ-sarcoglycan, β-sarcoglycan, and

[45] *Jefferies JL, Towbin JA. Dilated cardiomyopathy. Lancet. 2010*

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[47] *Debold EP, Schmitt JP, Patlak JB, et al. Hypertrophic and dilated cardiomyopathy mutations differentially affect the molecular force generation of mouse alpha-cardiac myosin in the laser trap assay. Am J Physiol Heart Circ Physiol 2007*

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[49] *Lakdawala NK, Funke BH, Baxter S, et al. Genetic testing for dilated cardiomyopathy in clinical practice. J Card Fail. 2012*

metavinculin[50] [45]. Desmosome gene mutations are a known cause of arrhythmogenic right ventricular cardiomyopathy (ARVC), but may also play a role in DCM.
This kind of cardiomyopathy is a relatively common feature of several forms of inherited skeletal myopathies, including those caused by mutations in dystrophin (DMD) and desmin (*DES*)[51]. *SCN5A* gene, coding for cardiac sodium channel, Nav1.5, is more rarely affected by mutations causing DCM. DCM has few clinical characteristics that allow subcategorization, anyway the condition is sometimes classified as DCM with prominent conduction system defects and arrhythmia (DCM associated with mutations in DES, LMNA, or SCN5A), or DCM with neuromuscular defects[52]

All these possible genetic mutations and all the different associated pathogenic mechanisms could explain why patients shows a variety of phenotypes, including extra-cardiomyopathic phenotypes like atrioventricular (AV) block, interventricular block, atrial fibrillation but also skeletal myopathy or hearing loss.[53] [54] [55]. DCM diagnosis is dependent on patient history and clinical features; echocardiographic, or cardiac MRI features are fundamental for the diagnosis of dilated cardiomyopathy. Electrocardiography, another standard diagnostic test, can show sinus tachycardia, ST-T wave changes, Q waves, conduction disease, bundle-branch block, left ventricular hypertrophy, or ectopies, including supraventricular tachycardia, atrial fibrillation, or ventricular arrhythmias[56]. The prevention of sudden cardiac death (SCD) is a primary concern in patients with dilated cardiomyopathy. DMD is associated with SCD and heart failure, resulting in a large health burden because of the very high rate of hospital admissions and the potential need for heart transplantation[56]. Medical therapies, especially beta blockers and aldosterone antagonists, reduce the risk of cardiac arrest in patients with DCM and should be used in accordance with guideline recommendations[57]. ICD (Implantable cardiac defibrillator) is used to prevent SCD in patients with history of SCD or prior cardiac arrest and sustained ventricular

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[52] *Hershberger RE, Hedges DJ, Morales A. Dilated cardiomyopathy: the complexity of a diverse genetic architecture. Nat Rev Cardiol. 2013*
[53] *McNair WP, Sinagra G, Taylor MR, et al. SCN5A mutations associate with arrhythmic dilated cardiomyopathy and commonly localize to the voltage-sensing mechanism. J Am Coll Cardiol. 2011*
[54] *Schönberger J, Wang L, Shin JT, et al. Mutation in the transcriptional coactivator EYA4 causes dilated cardiomyopathy and sensorineural hearing loss. Nat Genet. 2005*
[55] *Nigro G, Comi LI, Politano L, Bain RJ. The incidence and evolution of cardiomyopathy in Duchenne muscular dystrophy. Int J Cardiol. 1990*
[56] *Jefferies JL, Towbin JA. Dilated cardiomyopathy. Lancet. 2010*
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tachycardia, but can also be used in primary prevention in patients with severe systolic dysfunction (ejection fraction <30%)[58].

DCM could also be a x-linked pathology, associated with two principal disorders: one occurs in adolescent or young adults (see below), one is identified in babies and children, associated with Barth syndrome. Towbin and collaborators first identified the gene responsible for the x-linked DCM presenting in teenage males; studying two families affected by the x-linked cardiomyopathy, they found a strong linkage with alterations in dystrophin gene expression in the heart[59]. These patients show a rapid progression to heart failure and required heart transplantation if they were males, while female carriers showed a late onset, around the fifth decade, and a slower progression to heart failure[60]. Total absence or reduced expression of dystrophin are also related to muscular dystrophy like Duchenne or Becher muscular dystrophy (DMD, BMD), in which there is a progressive loss of muscular straight, since infant age, and affected are wheelchair bound by 11–12 years of age; cardiac manifestation includes, indeed, DCM and arrhythmias evident after 10 years of age[61]. Dystrophin is part of the Dystrophin-Glycogen Complex (DGC), a protein complex that provide a linkage between the cytoskeleton and the extracellular matrix and plays an important role in force transduction and cell signalling[62]. Other important evidence of the role of dystrophin in heart function and DCM progression is the high levels of creatine kinase (CK) found in both, patients with DMD and patients with x-linked DCM. Those information about the role of dystrophin suggest a high implication of cytoskeleton and sarcolemma alterations in DCM progression[63] [64].

[58] *ACC/AHA/HRS 2008 Guidelines for Device-Based Therapy of Cardiac Rhythm Abnormalities: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the ACC/AHA/NASPE 2002 Guideline Update for Implantation of Cardiac Pacemakers and Antiarrhythmic Devices) developed in collaboration with the American Association for Thoracic Surgery and Society of Thoracic Surgeons.* J Am Coll Cardiol. *2008*

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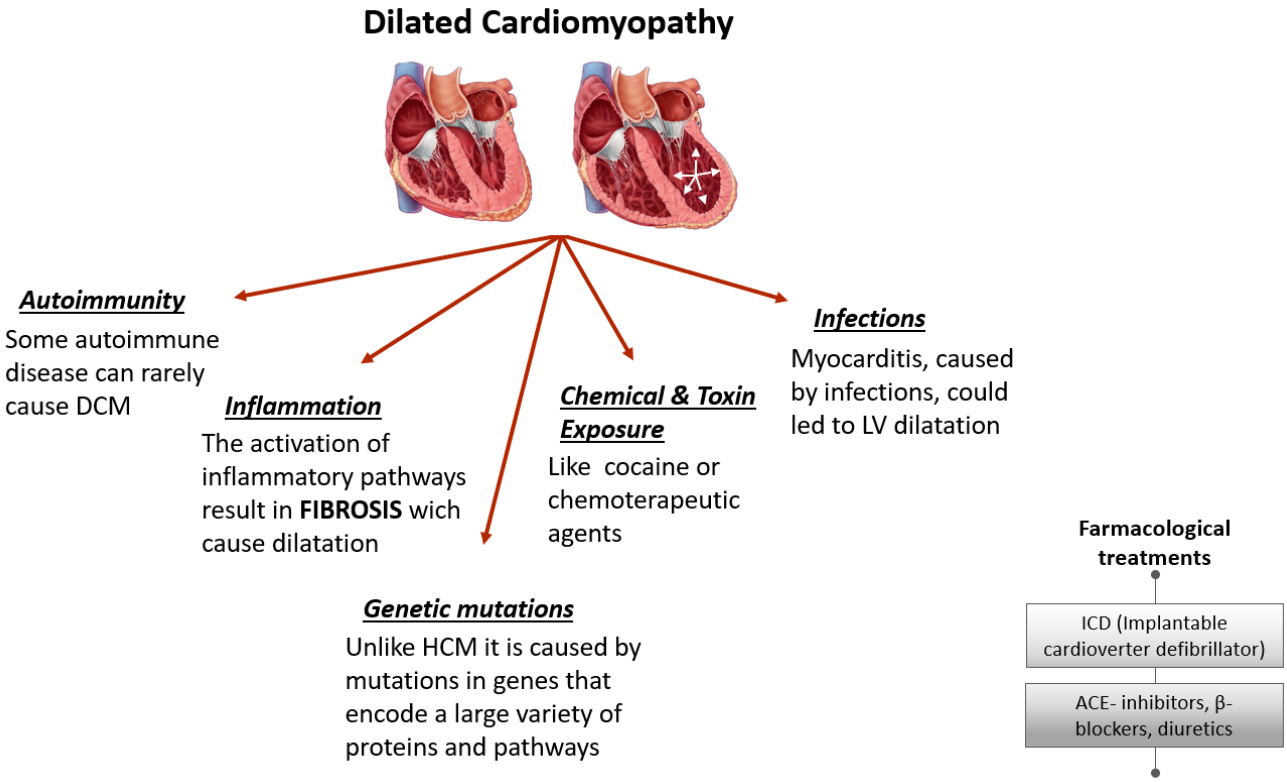

Figure 3: DCM remodelling, possible causes of disease and pharmacological treatment

In the picture a brief summary of the possible causes of DCM and its typical anatomical alteration. DCM is characterized by a dilatation of the ventricular chamber and it could be the consequence of a genetic mutation, of an autoimmune disease or inflammation but could also be secondary to toxin exposure or infection. There is no specific treatment for the disease, only the implantation of an ICD is recommended in the most serious cases, otherwise ACE-inhibitors, β-blockers or diuretics could be prescribed.

Adapt from Dilated cardiomyopathy Dilated cardiomyopathy. Nat Rev Dis Primers (2019)

Ion channel dysfunctions: Channelopathies are currently considered as disorders of the cardiomyocytes, and are listed among genetic cardiomyopathies. In particular, we will focus on *SCN5A* mutations. Inherited cardiac channelopathies are caused by mutations affecting genes encoding membrane ion channels (Sodium, potassium and calcium channels)[65]. Those mutations can disrupt the fine balance of ionic currents that shape the action potential and could therefore cause life-threatening arrhythmias in the absence of structural heart defects[66]. Channelopathies, such as long QT syndrome (LQTS), Brugada syndrome (BrS), short QT syndrome (SQTS), and catecholaminergic polymorphic ventricular tachycardia (CPVT) are characterized by malignant arrhythmias in a normal heart, resulting from genetic alterations in ion channels or associated proteins that could, as a consequence, cause sudden cardiac death[67].

Brugada syndrome was reported as "sudden unexplained nocturnal death syndrome" characterized by nocturnal death primarily in males around 40 years of age[68]. It is clinically characterized by ST segment elevation and it is responsible for 4 to 12% of all sudden death cases in young patience. The first genetic alteration associated with BrS was identified in the *SCN5a* gene encoding the α-subunit of the cardiac sodium channel, Nav1.5, and it was a "loss of function" mutation[69]. Since this first discovery, more than 450 pathogenic variants have been identified in 24 genes encoding sodium, potassium, and calcium channels or associated proteins, such as those involved in channel trafficking. There are two theories explaining how mutations trigger arrhythmias in this pathology: the repolarization hypothesis, which considers transmural dispersion of repolarization as the key substrate for re-entrant arrhythmias, and the depolarization hypothesis in which conduction abnormalities (atrio-ventricular conduction delay) are considered responsible for arrhythmias occurrence. However, BrS phenotype might be caused by many pathophysiological mechanisms, and the same mechanism might not be responsible for the disease in all patients[70]. Recently, the failure of familial-linkage analysis in uncovering new disease-causing genes, have suggest a more complex inheritance.[71] Patients affected by Brugada syndrome are susceptible to arrhythmias vagal triggered, so those patients are at risk of SCD during rest, rarely during exercise[72].

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[70] *Sieira J, Dendramis G, Brugada P. Pathogenesis and management of Brugada syndrome. Nat Rev Cardiol. 2016*

[71] *Bezzina CR, Lahrouchi N, Priori SG. Genetics of sudden cardiac death. Circ Res. 2015*

[72] *Kuriachan VP, Sumner GL, Mitchell LB. Sudden cardiac death. Curr Probl Cardiol. 2015*

Long QT Syndrome (LQTS) was first characterized 70 years ago in a family where 3 out of 5 children died suddenly during infancy. All of them had a prolonged QT interval (QTc values >470 ms for males and >480 ms for females) but no other signs of heart disease. This condition, called long QT syndrome could be congenital or acquired (for electrolyte imbalance, the effects of drug, ore the consequence of structural diseases). The congenital form is caused by mutations in ion channels genes. The clinical manifestations of LQTS can be variable, ranging from asymptomatic patients diagnosed through family screening, to SCD, syncope, convulsions, malignant ventricular arrhythmias, VF, and, typically, torsade de pointes[73]. Mutations in genes that encode for ion channel subunits lead to prolongation of the action potential either by reducing outward potassium currents or by increasing inward sodium or calcium currents. This evidence supported the classical view that the occurrence of early after depolarisations, associated with the prolongation of action potential, lead to torsade des pointes. This type of arrhythmia is typical of LQT syndrome and helps making the correct diagnosis[71]. Sudden death is a quite common condition in those patients, and usually occurs in healthy children and teenagers in physically or emotionally stressful situations[74]. The diagnosis is based on electrographic features and treatment is necessary to avoid SCD; usually the implantation of ICD is required. The three major LQTS-susceptibility genes are *KCNQ1*, *KCNH2*, and *SCN5A*, and mutations in these genes are associated with about 75% of patients with a clinical LQTS diagnosis. The genetic heterogeneity of LQTS has led to its classification into subtypes based on genetic loci. Gain-of function variants in *SCN5A* (LQT3) induce an increased late inward Nav1.5 currents that slow down cardiac repolarization, also causing a prolonged QT interval. LQT1 arise from dysfunction in *KCNQ1* gene, encoding for the α-subunit of Kv7.1 channel, mediating a slowly activating delayed rectifier potassium current (I_{Ks}); mutations in *KCNQ1* cause a reduction in I_{Ks} currents by modification in channel kinetics that determine a prolongation of AP and QT segment. LQT 2 syndrome is associated with a reduction of the I_{Kr} amplitude, determined by mutations in human ether-à-go-go related gene (hERG) or *KCNH2* that prolonged cardiac repolarization[75]. LQT4 is associated with mutation of Ankirin 2 Gene (ANK2), that reduce its activity; LQT7 is caused by loss of function mutation in *KCNJ2*. Many different mutations can cause LQT Syndrome, not only gain of function or loss of function, but the phenotype depends on which gene is involved and where the mutation is located[76]

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Short QT Syndrome (SQTS) is a rare inheritable cardiac channelopathy characterized by abnormally short QT intervals and an increased propensity to develop atrial and ventricular tachyarrhythmia in the absence of structural heart disease, Cardiac arrest seems to be the most frequent symptom (up to 40%)[77]. Currently, genetic alterations associated with SQTS have been identified in six genes encode for calcium and potassium cardiac channel.

Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) is a pathological condition whereby intense physical exercise or acute emotional stress can trigger abnormal heartbeat that can lead to dizziness, fainting (syncope), and in worst cases to cardiac arrest and sudden death. Syncope is the first clinical manifestation of CPVT patients and less prevalent signs and symptoms include dizziness or palpitations. Genes involved in pathogenesis are *RYR2* and *CALM1-2-3*[78].

SUDDEN CARDIAC DEATH AND INHERITED CARDIOMYOPATIES

SCD is described as the unexpected natural death from a cardiac cause within a short time period, generally ≤1 hour, from the onset of symptoms, in a person without any prior condition that would appear fatal[79]*;* such a rapid death is often attributed to a cardiac arrhythmia but only an ECG or a ventricular electrogram recorded from an implanted device at the time of death can provide definitive information about an arrhythmia[80]

SCD accounts for ≈20% of mortality in the general population and is associated with significant morbidity among survivors. It occurs in the setting of a broad spectrum of cardiac pathologies, and although it mostly occurs in adults, it may also strike children[81]. The major cause of SCD after the age of 45 years is coronary artery disease, but in the paediatric population and in young adults, SCD typically occurs in the setting of rare inherited cardiac disorders[82]. SCD accounts for 19% of sudden deaths in children between 1 and 13 years of age and 30% between 14 and 21 years of age[83].

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80 Zipes DP, Wellens HJ. Sudden cardiac death. Circulation. 1998

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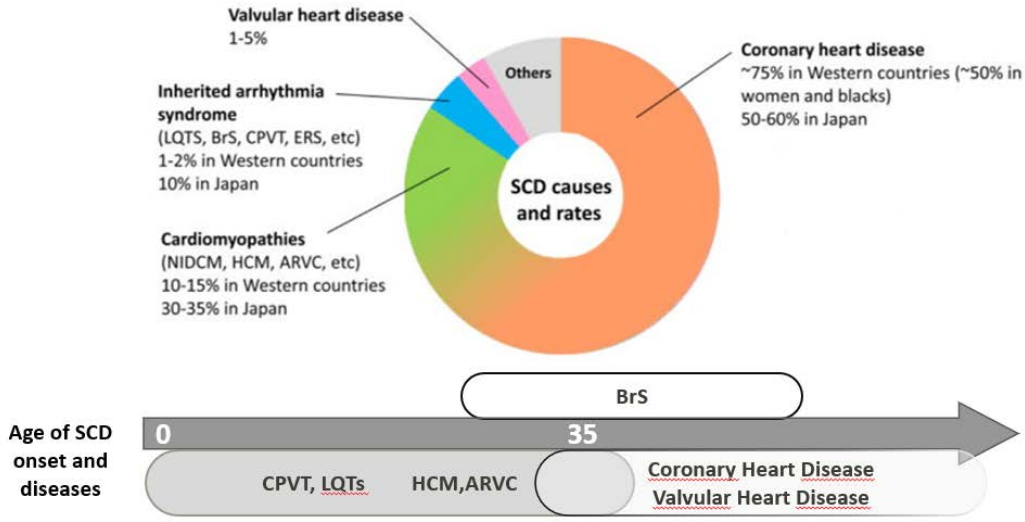

Figure 4: Causes of SCD rates and age of onset in each disease

Coronary heart disease is the prominent cause of SCD and the onset of sudden death is predominantly in elderly population; Cardiomyopathy are the second leading cause for SCD, in those cases the onset of sudden death is at age less than 35 years. ARVC indicates arrhythmogenic right ventricular cardiomyopathy; BrS, Brugada syndrome; CPVT, catecholaminergic polymorphic ventricular tachycardia; ERS, early repolarization syndrome; HCM, hypertrophic cardiomyopathy; LQTS, long QT syndrome; NIDCM, non-ischemic dilated cardiomyopathy; SCD, sudden cardiac death[84].

Ventricular arrhythmias, both monomorphic and polymorphic, are the most common causing events. However, atrial tachyarrhythmias with rapid atrioventricular (AV) conduction degenerating into ventricular fibrillation (VF), have also been described, as well as bradyarrhythmic deaths due to AV block[85]. The occurrence of SCD is an interplay between the anatomic and functional substrates, modulated by transient factors (abnormalities in electrolytes, changes in pH or PO_2, influence of the peripheral neurophysiological actions etc.[86]) that perturb the cardiac electrical balance. It is absolutely hard to predict the occurrence of SCD, because of the many events that must happened together to generate lethal arrhythmias; but there are some conditions, such as inherited cardiomyopathies, in particular those caused by specific mutations, which make carriers more susceptible.

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[85] *Ávila P, Chaix MA, Mondésert B, Khairy P;" Sudden Cardiac Death in Adult Congenital Heart Disease" Card Electrophysiol. clin 2017*

[86] *Zipes DP. "Unwitting exposure to risk." Cardiol Rev.1993*

If SCD occurs in the absence of coronary artery disease or heart failure, the main cause is HCM[87]; HCM predisposes to SCD due to myocardial hypertrophy, myocyte disarray and fibrosis. Apart from direct pro-arrhythmia effects, these changes can lead to SCD secondary to myocardial ischemia, diastolic dysfunction, left ventricular outflow obstruction and the development of congestive heart failure[88]. Incidence of SCD in HCM patients decreases into older adulthood; current therapies are improving survival among young HCM patients[89]. Guidelines to prevent SCD in HCM patients are not universally accepted and differ from USA to Europe: the wall thickness, family history of SCD and left ventricular outflow tract gradient, are some of the parameters use to determine the risk of SCD in this population and the need to implant ICD. Anyway, prevention of SCD is obtained also thanks to pharmacological treatments like the administration of beta-blockers and Disopyramide or calcium channel blockers and many other novel agents in study.

DCM is also often associated with SCD; here SCD is most frequently caused by ventricular tachyarrhythmia (approximately 50%) or ventricular fibrillation, but can also be due to bradycardia and electromechanical dissociation. The incidence of SCD in DCM has been drastically reduced by treatment with beta blockers, ACE inhibitors and other drugs. Implantable cardiac defibrillator (ICD) should be considered as primary prevention as guidelines suggest[85].

In about 5%–10% of cases of SCD, no underlying heart disease can be found at autopsy; those are probably the ones with genetic disorders that lead to ion channel dysfunctions[90]. Brugada syndrome is one of these, accounting for 20% of SCD in patients with normal hearts, usually occurring during rest, sleep or fever[91]. The mutations responsible of BrS generate a transmural gradient that predisposes to re-entry which, in turn, causes short-coupled ventricular premature beats, polymorphic ventricular tachycardia and ventricular fibrillation, the latter being trigger for premature SCD[92]. Br syndrome displays an incomplete age and sex-related penetrance, with an autosomal dominant pattern of inheritance; in contrast with HCM, most lethal events have been

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[92] *Antzelevitch C. Brugada syndrome. Pacing Clin Electrophysiol. 2006*

identified in men after the 4th decade of age[93] [94] however, Brugada syndrome patients affected by loss of function mutations in *SCN5A* gene may experience SCD in childhood[95].

LQTS is another channelopathy related to high risk of SCD; it is caused by mutations in different sodium and potassium channels leading to a prolongation of QT segment. The characteristic ventricular arrhythmia in LQTS is Torsade de Pointes, typically triggered by a short-long-short cycle, that could culminate in SCD[96]. Considering all the different phenotypes and genotypes of LQTS, we could not find any significant difference between the QT interval prolongation within the different LQT syndromes, anyway multiple ST-segment and T-wave morphologies have been associated with particular genotype and risk of SCD[97] indeed, different type of LQTs are susceptible to different triggers of sudden death on the base of ST-segment and T wave morphology; for instance, LQT2 syndrome patients have low amplitude T-waves and more often sudden cardiac death occurs as reaction to strong emotions, while LQT3 syndrome patients, with a late-peaked T-wave and flat ST segments, are at high risk of arrhythmias during bradycardia, and have a higher incidence of SD during sleep[98] [99].Moreover LQT1 and 2 usually show some syncope events that warn patients and doctors before SCD occurs, while LQT3 could appear with SCD directly without any prior symptoms. In LQT1 syndrome, the highest risk is in boys between 5 and 15 years of age who practice swimming or intense activities, because its typical tract is a broad T-wave. In LQT 2 syndrome the higher risk of SCD is in women up to 9 months post-partum and arrhythmias are linked to the low amplitude of the T-wave notch. Subjects affected by those two syndromes should avoid auditory or emotional stimuli and nocturnal arrhythmias are uncommon. As already said, LQT3 syndrome leads carriers to bradycardia and patients are susceptible to SCD during rest or night sleep[100].

By definition, SCD, is unpredictable and unexpected, especially in young adults. Optimal management of non-ischemic cardiomyopathy patients includes administration of guideline directed medical therapy. Between the available therapeutic options, implantable cardioverter defibrillator (ICD), that has the ability to treat lethal ventricular arrhythmias, is one of the first

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recommendations to avoid SCD in susceptible patients[101]. Susceptibility is determined by the reduction of the left ventricle ejection fraction that should be less than 35%[102] but these criteria do not consider a subgroup of patients with normal or mildly reduced ejection fraction but still at high risk for SCD. Since the current guidelines fail to identify all the high-risk patients, new biomarkers and criteria should be investigated[103]. That's why many groups are trying to identify some blood biomarkers to predict SCD occurrence (markers of myocardial stress, injury, inflammation or remodelling)[104]; some are trying to use the presence of some evidence, like myocardial scarring, identified with magnetic resonance imaging (CMR), to predict the risk of SCD[105]. Not only the ICD could be protective from SCD, also many pharmacological strategies could avoid the occurrence of sudden death, but effectiveness depends on pathology and on the phenotype manifested in patients.

[101] *Al-Khatib SM, Stevenson WG, Ackerman MJ, et al. "2017 AHA/ACC/HRS guideline for management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: Executive summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines and the Heart Rhythm Society." Heart Rhythm. 2018*

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CARDIAC EXCITABILITY AND ARRHYTHMIAS

Human cardiac excitation starts from the sino-atrial node and propagates through the atria, the atrioventricular node, the His-Purkinje fibres and ventricles. Any abnormalities in the heart rhythm, in its propagation or in its generation, leads to cardiac arrhythmias. In the heart, tissue specific types of action potentials are described, their sum giving rise to the typical ECG registration. They all have in common 5 phases: phase 0 or rapid depolarization thanks to the opening of the fast sodium channel; phase 1 or rapid repolarization, caused by the inactivation of sodium channels and the openings of fast potassium channels (I_{TO}), that ends with the opening of calcium channels; phase 2 where there is an equilibrium between ion fluxes, calcium, sodium and potassium, generating a plateau phase responsible for the duration of the action potential; phase 3 that starts when calcium and slow sodium channels close and repolarizing potassium currents prevail, bringing the membrane potential back to the resting voltage; phase 4 is the maintenance of the resting voltage, where resting potassium currents keep the membrane potential at negative voltages. Nodal cells have a different resting potential, more positive than the working myocardium and it has a tendency to spontaneous depolarization, until it reaches the threshold voltage to open the calcium channel and trigger a new action potential; the phase 4 of nodal cells is shorter than in working myocardium. Nodal cells also have a prolonged refractory period to slow down signal propagation between atria and ventricles allowing the ventricles to be filled before systole occur. Nodal cells are responsible for the generation and propagation of the action potential, Purkinje fibres propagates the electrical signal through the heart while myocardium cells trigger contraction.

Role of Ca^{2+}, K^{+} and Na^{+} in action potential generation and excitation-contraction coupling in the myocardium

At molecular level, while nodal cells are responsible for the heart rhythm and generate the electrical stimulus that excite myocardium and triggers contraction, working cardiomyocytes are responsible of transforming the electrical stimulus to contraction. This phenomenon is called excitation-contraction coupling and it occurs in cardiomyocytes every time cells receive an electrical stimulus and generate an action potential (AP). The ions responsible for cardiac excitation-contraction coupling are potassium, sodium and calcium. AP in the myocardium, starts with the opening of Na^{+} voltage sensitive channel (Nav1.5) on cardiomyocytes membrane (phase 0); the increase of intracellular Na^{+} concentration causes a rapid depolarization that open L-Type Ca^{2+} channels (Cav1.2, LTCC) (ends Phase 1). This small amount of calcium, accumulating around the cytoplasmic side of the channel, leads to a larger release of calcium from the SR through Ryanodine-receptor type 2 (RYR2) channels, located on the SR membrane. Now, the increment of the intracellular Ca^{2+} concentration is huge and makes contraction possible thanks

to the binding between the regulatory calcium sensing protein of the contractile apparatus, cardiac Troponin C (cTnC), and the bivalent ion now largely available in the cytosol. When Ca^{2+} binds cTnC, it changes the protein conformation, this alteration causing the sliding of the tropomyosin on the actin filaments so that the sites for myosin head interaction become free and the cross bridges could be formed. During diastole, relaxation occurs when cytoplasmic Ca^{2+} concentration is reduced; reduction occurs thanks to Ca^{2+} ATPase ion pump SERCA2a on the sarcoplasmic reticulum membrane, which is usually inhibits by Phospholamban (PLB). The high cytosolic Ca^{2+} concentration activates the sodium calcium exchanger (NCX1) in forward mode, which imports 3 Na^{+} and extruded 1 Ca^{2+} ion, and the Calcium calmodulin dependent Kinase II (CAMKII), that has different targets such as sodium channels, calcium channels and RYR2. Also, PKA is activated during contraction and PLB is one of its targets; when phosphorylated, it removes the block from SERCA and contributes to the reduction of cytosolic calcium concentration. During diastole, the inactivation of L-type Ca^{2+} currents and the activation of SERCA2a brings the calcium concentration back to lower levels, so the ion detaches from cTnC, the cross-bridge interaction is stopped and relaxation occurs. At the same time, the activation of potassium repolarizing currents brings the membrane potential back to resting voltages and the contraction ends with the end of AP[106].

Increases in $I_{Ca\text{-}L}$ or in $I_{Na\text{-}L}$ cause the prolongation of the action potential duration (APD) and SR Ca^{2+} overload[107] while AP prolongation provides a substrate for early afterdepolarizations (EADs), spontaneous Ca^{2+} release from the overloaded SR, induces delayed afterdepolarizations (DADs). As already said both EADs and DADs, are triggers for re-entry and sustained cardiac arrhythmias[108].

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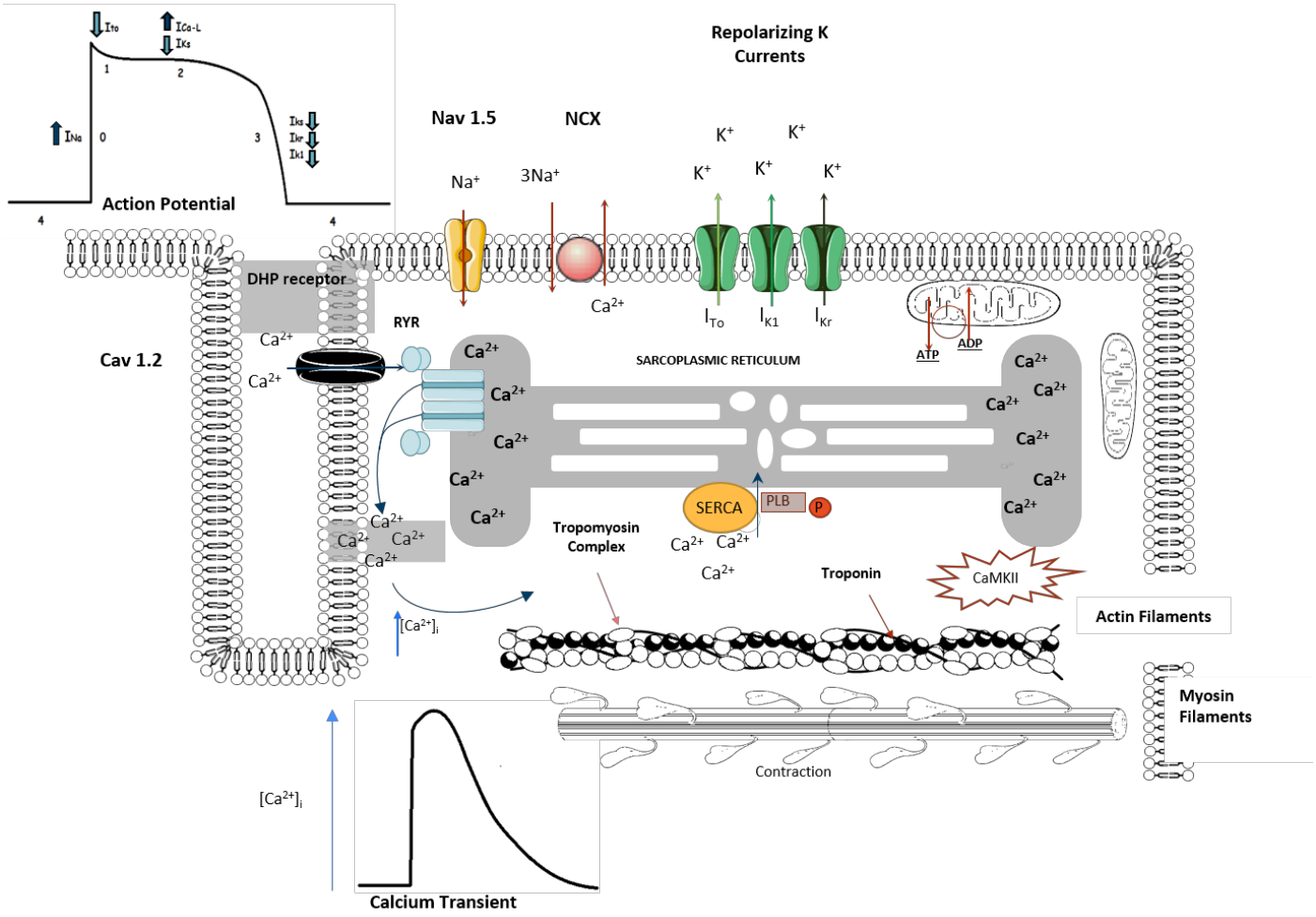

Figure 5: Graphic representation of structures involved in the cardiomyocyte's contraction

On top, the action potential with currents responsible for the different phases. In the cartoon the schematic representation of a cardiomyocyte; the sarcoplasmic reticulum (SR), in the centre, is the stock of calcium ions (Ca^{2+}), associated on the left with the ryanodine receptor (RYR) which interact with the calcium channel Cav1.2 on the cell surface. When a small amount of Ca^{2+} passes through the open Cav1.2 and accumulates around the RYRs, those open the channel, allowing calcium to escape from the SR, thus allowing the bivalent ion to bond with tropomyosin, unblocking myosin's binding sites on actin filaments and ensure contraction. SR is also associated with SERCA, the ion pump for calcium reuptake usually blocked by phospholamban (PLB). SERCA is activated after PLB phosphorylation by PKA and is responsible for the reduction of $[Ca^{2+}]$ I and of diastole. The calcium transient represents the trend of calcium concentration in the cytosol corresponding to the cell contraction. On top, in the cell membrane, from left to right are represented the fundamental ion channels, Nav1.5 responsible for the rapid inward sodium currents that makes the rapid depolarization of the cardiomyocyte's membrane, then the sodium calcium exchanger which import 3 Na^+ and extruded 1 Ca^{2+} ion and in the end the repolarizing potassium channels responsible for the repolarizing currents.

Cardiac arrhythmias mechanisms

The mechanisms responsible for cardiac arrhythmias are generally divided into two major categories: (i) enhanced or abnormal impulse generation (i.e., focal activity) and (ii) conduction disturbances (i.e., re-entry)[109] . The first includes the enhanced automaticity that could occur with some heart diseases; the most common arrhythmia events caused by abnormal impulse generation are early after depolarizations (EADs) and delayed after depolarizations (DADs)[110] *(Fig.5)*

EADs are depolarizations that interrupt or delay repolarization during phase 2 or 3 of the action potential (AP)[111]. They are typically observed in injured heart tissue. Usually, EADs occur when the altered balance of active currents during phase 2 or 3 shifts towards the inward depolarizing direction and determines a prolongation of the action potential duration (APD)[112]; the causes of the APD prolongation could be a reduction of repolarizing potassium current ($I_{Kr,}$ $I_{Ks,}$ I_{K1}), an increase in late calcium current (I_{CaL}), increase of NCX forward activity (3 Na^+ in vs 1 Ca^{2+} out), or an increase of late sodium current (I_{NaL})[113] [114]. At membrane potentials negative to the threshold of I_{CaL} activation, (-30, -40 mV) spontaneous SR Ca^{2+} release-activated NCX favours the non-equilibrium reactivation of I_{Na}, driving phase-3 EADs induction[115] [116]. When cytosolic Ca^{2+} concentration remains high during repolarization, NCX can be activated in forward mode to extruded calcium, thus leading the membrane towards depolarization. If this mechanism occurs in condition of AP shortening, EAD onset is facilitates[117] [118].

DADs typically occur during diastole and in conditions of elevated cellular Ca^{2+}-loading[119]. The central event causing DADs is abnormal spontaneous release of Ca^{2+} from the sarcoplasmic reticulum during diastole; the release activates NCX in forward mode causing a net inward flow

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of positive ions, that depolarizes the cell[120]. Leak of calcium from SR could be because of abnormalities in RYR2 channels or because of calcium overload in the SR[121]. A contributor to calcium overload in the SR is the PKA dependent hyperphosphorylation of phospholamban (PLB) that leads to an enhancement of SERCA2a activity. The amplitude of the DAD depends on the size of the resting K^+ conductance, mainly determined by I_{K1}. When I_{K1} is low, the same I_{NCX} will produce a larger DAD and vice versa[122].

Re-entry is a circular movement of electrical propagation occurring when an activation wave-front propagates around an anatomic or functional obstacle or core, and re-excites the site of origin[123]. The main functional determinants of re-entry arrhythmias are myocardial refractory period (primarily driven by APD and by the recovery kinetics of I_{Na}), excitability, and conduction properties including conduction velocity heterogeneity and block[124]. Conduction velocity is mainly determined by excitatory Na^+ current, cardiomyocyte electrical coupling through gap junctions, and muscle bundle architecture[125]. Reduced I_{Na}, decreased gap junctional coupling, and muscle bundle discontinuities resulting from fibrosis reduce conduction velocity and may promote re-entry[126]. The re-entry-promoting substrate can be caused by disease-related cardiac remodelling or predisposing genetic factors, but can also be produced by altered restitution dynamics and subcellular Ca^{2+}-alternans (SR Ca^{2+} load and release alternans)[127]. Altered Ca^{2+} signalling can contribute to the formation of a re-entry substrate by two mechanisms: promoting dispersion of excitability, and promoting dispersion of refractoriness[128] DADs that do not reach the threshold to trigger an AP can cause depolarization by increasing Na^+-channel inactivation and promoting dispersion of excitability; EADs that remain below the threshold to propagate may increase dispersion of refractoriness, also creating a re-entry substrate[129].

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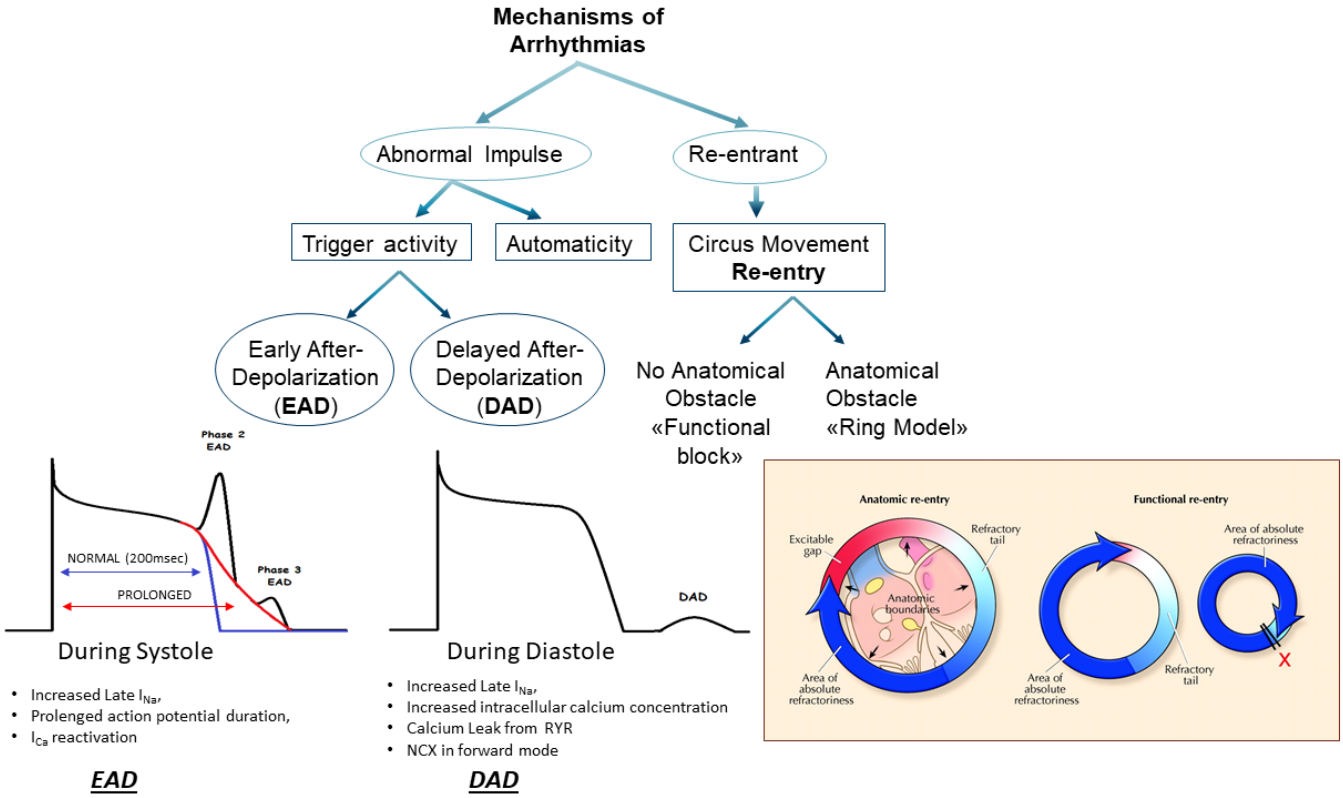

***Figure 5:** Typical mechanisms of arrhythmias*

Following the classical division into two major categories, we can divide arrhythmias mechanisms as abnormal impulse and a re-entrant circus movement. The abnormal impulse is the mechanism behind the generation of trigger activity like early and delayed after depolarization (EAD and DAD respectively) described in the left part of the figure; here are also mentioned the main pathological mechanisms determining the abnormal impulse and the onset of DAD or EAD during systole or diastole. On the right side the re-entrant mechanism is describe; it can occur for the presence of an anatomical obstacle or for the presence of a functional obstacle to the propagation of the electrical signal.

Arrhythmias and their occurrence within the main cardiomyopathies

In channelopathies like Brugada syndrome or LQT syndrome (in particular Type-3 will be of particular interest for us), mutations on genes encoding for ion channels lead to abnormalities of excitation contraction coupling, while in other cardiomyopathies like hypertrophic cardiomyopathy, arrhythmias occur as a result of a large remodelling of the heart tissue that secondary leads to alterations of ion channels function.

In Brugada syndrome (BrS), the mechanisms of arrhythmogenesis can be explained by the heterogeneous shortening of APDs in the right ventricular epicardium[130]. BrS has been associated with mutations in 7 different genes. Most common mutations are in SCN5A (Nav1.5, BrS1), 11% to 28% of BrS probands, CACNA1C (Cav1.2, BrS3) in 6.7%, CACNB2b (Cavb2b, BrS4) in 4.8%[131] [132]. An outward shift in the balance of currents that are active during phases 1 and 2 of the epicardial action potential via either, a reduction of inward current (I_{Na} or I_{Ca}) or an increase in outward current (I_{Kr} or $I_{K\text{-}ATP}$), allows the already prominent I_{to} to accentuate phase 1 repolarization. When phase-1 is repolarized beyond the voltage range at which L-type Ca^{+2} channels activate, the Ca^{+2} channels fail to activate, resulting in loss of the action potential plateau, predominantly in the right ventricular subepicardial cells where I_{to} is most prominent[133]. Loss of plateau cause a transmural (as well as epicardial) dispersion of repolarization. The transmural dispersion contributes to development of ST segment elevation and the generation of a vulnerable window across the ventricular wall; those leading to re-entry arrhythmias that precipitates in ventricular tachycardia (VT) or ventricular fibrillation (VF)[134]

LQT3 syndrome is characterized by "gain of function" mutations in *SCN5A* gene or channel interacting proteins, that lead to an increase of I_{Na} and/or $I_{Na\text{-}L}$ [135]. An increase in $I_{Na\text{-}L}$ means prolonged APs and may provide both a substrate (by enhancing the spatio- temporal dispersion of ventricular repolarization and refractoriness, which lead to conduction block and re-entrant activity) and triggers (EAD-and DAD-s, triggered activity and increased automaticity) that, increase the probability of torsades de pointes. Those arrhythmias can progress to ventricular fibrillation and to syncope, cardiac arrest and SCD[136].

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The effects of the increased I_{Na-L} are to increase Na^+ influx during the AP plateau, causing AP prolongation and an increase of the reverse mode of NCX activity (Ca^{2+} influx with Na^+ efflux) due to Na^+ overload. The rise of both Na^+ and Ca^{2+} concentrations, promote increased cellular Ca^{2+} entry and thereby Ca^{2+} up-take by the sarcoplasmic reticulum Ca^{2+}-mediated activation of CaMKII leading to calcium overload and promoting DADs and EADs[137].

In HCM, we observed a prolongation of APD associated with mild prolonged QTc[138]. Prolonged APD was the main determinant of the increased risk of early afterdepolarisations (EADs)[139]. Interestingly, the frequency of EADs and the degree of APD prolongation went hand in hand with the incidence of ventricular arrhythmias in patients[140].

APD prolongation in HCM cardiomyocytes is caused by a combination of decreased repolarizing potassium currents (I_{to}, I_{K1} as well as delayed rectifier K^+ currents) and increased depolarizing (Ca^{2+} and Na^+) currents. Reduced density of potassium currents is the consequence of the lower levels of expression of K^+ channel genes and this might be a consequence of the increased activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[141]. The inactivation kinetics of I_{CaL} is markedly slower in HCM cardiomyocytes[142] [143] and I_{NaL} was consistently increased in all studied HCM myocytes. Changes in Ca^{2+}, late Na^+ and K^+ current densities are sufficient to explain the prolongation of APD in human HCM cardiomyocytes[144]

In human HCM cardiomyocytes, abnormalities of APD and ion current were paralleled by marked alterations of intracellular Ca^{2+} handling. Ca^{2+} overload is the consequence of an increased Ca^{2+} entry via L-type Ca^{2+}-current [due to prolongation the action potential (AP) plateau], combined with a reduced rate of Ca^{2+}-extrusion through the Na+/ Ca^{2+} exchanger [due to increased cytosolic (Na^+)] and a lower expression of SERCA. Increased late Na^+ current (I_{NaL}) plays a major role, as it causes both AP prolongation and Na^+ overload[145]. The increase of $[Ca^{2+}]_i$ is likely to be a direct consequence of some of the sarcomere mutations that cause the disease

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but arrhythmias also depend on many remodelling processes involved in HCM, like fibrosis and loss of T-Tubules. Accumulation of intra-myocardial fibrosis and replacement scars underlies heart failure progression and represents a substrate for sustained arrhythmias[146]. On the other hand, T-tubule depletions has also its effects; indeed, the loss of t-tubules may directly contribute to slow down the kinetics of Ca^{2+} transients[147], thus delaying relaxation, with possible detrimental effects on diastolic function. Loss of T-tubules reduces synchronicity of Ca^{2+} release, thus raising the likelihood of APD and effective-refractory-period (ERP) temporal fluctuations (alternans): APD and ERP alternans facilitate the formation of dynamic re-entry circuits[148].

Arrhythmias in HCM can't be the sole consequence of the tissue remodelling, the presence of extended fibrotic scar tissue and structural alteration make the substrate for re-entry, but ectopic premature activations are essential to initiate arrythmias, and alterations in ion currents and ion handling in cardiomyocytes give rise to the essential triggers to initiate the re-entry. All those remodelling processes described lead to early- and delayed- afterdepolarizations, which are the cellular arrhythmic events that may result into the spontaneous generation of premature APs in the affected cardiomyocytes[149]. The evidence that SCD and arrhythmic events occur in the early phase of HCM disease[150] [151], when the tissue remodelling has yet to come, highlights the role of EADs and DADs and the electrical cellular remodelling in giving rise to ventricular fibrillation and a re-entry substrate.

In dilated cardiomyopathy, the mechanisms behind the insurgence of lethal arrhythmias are not clear as many genetic driven mechanisms, of remodelling and cellular dysfunction, occur together, possibly leading to ventricular arrhythmias, heart failure or similar phenotypes at rest or during exercise. Those phenotypes could be the consequence of alterations in calcium handling because of mutations in sarcomeric proteins or in genes encoding for cytoskeletal proteins. Mutations in sarcomeric genes indeed perturb the actin-myosin interaction and force generation and may disrupt Troponin C calcium sensitivity or cause the loss of sarcomeric organization[152]

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[153]. In children, also metabolic abnormalities or mitochondrial function alteration could have a causal role in developing diastolic disfunction and alteration of right ventricular function[154].

Antiarrhythmic drugs

Arrhythmic events in cardiomyopathy are common events and are one of the main risk factors for SCD occurrence. They depend primarily on ion current alterations and altered ion homeostasis, before tissue remodelling and fibrosis, therefore treatment includes drugs to regulate ion currents and restore the conduction system. The management of those events became of primary interest in the clinical practice to avoid sudden death. Antiarrhythmic drugs include beta-blockers, calcium antagonists, negative inotropic agents and sodium channel blockers that are generally use to manage the insurgence of arrhythmic events like atrial fibrillations. In the clinical practice, the genetic background and the altered mechanisms responsible for the higher risk of arrhythmic events, drive the selection of the right therapy for each patient. Each cardiomyopathy patient needs to be treated specifically, because of the high variability in the genetic background of the disease and the different pathogenetic mechanisms behind the phenotype. Guidelines has the role of guiding the clinicians in the choice of the correct therapy to manage cardiomyopathies and the risk of arrhythmic events. Antiarrhythmic drugs are rather safe but alterations of the cardiac conduction system could also modify the responses to drug therapy, turning their antiarrhythmic effect in a pro-arrhythmic effect, thus causing adverse events.

β-blockers are one of the first-line therapy for the management of patients who need lifelong treatment for ventricular arrhythmias or for high SCD risk. Those drugs block the sympathetic system mediated triggering mechanisms of arrhythmia, slowing the heart rhythm and inhibiting the spontaneous or excessive calcium release from SR. β-blockers are a generally safe drug.

Class IA antiarrhythmic drugs include disopyramide and quinidine, which act as sodium channel blockers with an inhibitory effect on delayed rectifier potassium currents; thus, prolonging the QT interval that is why they are not recommended in LQTS. Class IB e IC antiarrhythmic drugs are sodium channel blockers able to block both the peak sodium current and the late sodium current. They include Mexiletine and Flecainide, and their ability to block the late sodium current makes them able to reduce the QT segment, so they are useful in LQTS treatment, especially in the type 3, caused by a gain-of-function mutation in Nav1.5 and an increase sodium current.

Calcium channel blockers (CCBs) are molecules capable of blocking the voltage-sensitive long-lasting calcium channel, reducing the heart rate and the calcium release from SR, promoting

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relaxation and increasing the refractory period duration. CCBs are suggested in those patients who are intolerant to β-blockers; Verapamil effectively reduced the left ventricle mass, improving diastolic filling, and reduces the obstruction on the left ventricle outflow tract at rest. Diltiazem shows a similar effect in restoring the diastolic function[155] [156].

As we previously said, tissue remodelling plays an important role in the insurgence of arrhythmias in cardiomyopathy, thus the use of drugs blocking or reversing the remodelling mechanisms could be a good perspective for the reduction of the incidence of SCD and arrhythmic events. These drugs, including ACE inhibitors, angiotensin II receptor blockers (ARBs) and mineral corticoid receptor antagonists (MRAs), are currently being evaluated.

We will briefly analyse the main pharmacological treatments in use to prevent arrhythmias in cardiomyopathies.

For DCM, the first line therapy is neurohormonal antagonists and implantable device therapy. These agents were demonstrated to be effective in reducing all-cause mortality in unselected adults with DCM. The main causes of death in DCM patients are HF and SCD secondary to AF and bradyarrhythmia. Usually, the treatment includes angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARB), beta adrenoreceptor blockers (BB), aldosterone antagonists, and vasodilators. Those treatments could be also associated or substituted by implantable cardiac devices, which is advised as primary prophylaxis in selected patients who are expected to survive in a good status for more than a year; usually, the recommendation for ICD increases with the decrease of systolic function; patients with an ICD who have experienced of recurrent shocks are usually advised to take amiodarone too.

HCM affected patients are encouraged to avoid competitive sports and intense physical activity. Patients could be divided in those with Left ventricle outflow tract obstruction (LVOTO) or without LVOTO; the first group could be treated with Disopyramide and β-blockers. Disopyramide is an antiarrhythmic drug (class IA), able to reduce the basal left ventricle outflow pressure gradients, to improve exercise tolerance and functional capacity without significant proarrhythmic effects. When β-blockers are non-tolerated, also calcium antagonists like Verapamil or Diltiazem could be used in substitution, but not dihydropyridine calcium antagonist. In LVOTO patients, invasive treatment should be taken into consideration to reduce the obstruction when the gradient at rest is higher than 50mmHg. In HCM patients without LVOTO, the aim is to reduce left ventricle diastolic pressure, to ameliorate LV filling and to slow the heart rate, so therapy includes β-blockers, calcium antagonists (non-dihydropyridine), with caution in

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the use of diuretics. Patients with reduced ejection fraction should be treated according to the ESC guidelines for chronic heart failure[157].

For the prevention of SCD, there are only few studies that show the efficacy of Amiodarone in reducing the incidence of SCD in HCM patients with ICD. For a secondary prophylaxis, in patients who survived to ventricular fibrillation and are at high risk for lethal arrhythmias, ICD is highly recommended to avoid[158] [159] [160] [161].

In patients with Brugada syndrome, ICD is not an optional treatment, but is the only therapeutic treatment available, and is recommended in subjects who experience spontaneous sustained ventricular tachycardia or survivors of an aborted cardiac arrest[162] [163].

For the LQT syndrome treatment, the choice depends on the type syndrome; QT-prolonging drugs are to be avoided, the ECG features and genetic background need to be taken into consideration to select the best treatment; LQTS patients who survived a cardiac arrest have a high risk of recurrence even if treated with β-blockers, that's why ICD is recommended in these patience[164].

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EXPERIMENTAL MODELS TO INVESTIGATE CARDIOMYOPATHIES

As previously described, cardiomyopathies are complex pathologies and have varied phenotypes and pathogenic mechanisms which makes them hard to model. There are several approaches to design cardiomyopathy models in a way that they best represent the pathology; these include (i) cell-based model system, mostly to study the effects of ion channels mutations on channel's kinetics and conductance, (ii) animal models, to study the multicellular effects of cardiomyopathies, (iii) human samples, which will be the best model of disease to test drug effects and to study the pathogenesis, and (iv) cardiomyocytes derived from human induced pluripotent stem cells. The latter are the latest discovery in basic science and are extremely useful in disease modelling and drug screening, because they reproduce the complete gene pool of the patient from whom they are derived.

The easiest way to understand dysfunctions at ion channels level is to reproduce the mutant channel in expression systems. Those systems have many limitations, from the lack of all the regulatory proteins usually involved in modulating channel function, to their distance from human cardiomyocytes in terms of cell shape, structure and function[165]. However, this expression systems have been an essential tool to understand the correlation between genotype and phenotype in Brugada syndrome, LQTs and to discover new features of channelopathies. BrS is caused by loss of function mutations in ion channels in the 11/28% of cases; 90% of those are mutations located in *SCN5A* gene (encoded for the α sub-unit of the cardiac sodium channel $Na_v1.5$)[166] . To clarify which features of the channel are affected by Brs-mutations, many studies employed expression systems; for example, in 2007 Dong-Jik Shin and co-workers designed plasmids with the sequences of the *SCN5a* gene, WT and with the new mutation they discovered in a BrS family; then they transfected the plasmid, together with those of the regulatory β sub-units, in tsA201 cells and finally analysed the channel kinetics and current intensity by using patch clamp technique. They discovered that peak I_{Na} was markedly reduced, which was the cause of BrS and of the SCD occurrence in the family[167]. Baroudi and co-workers expressed the $Na_v1.5$ channel (WT and with the G1740R mutation causing BrS) in a tsA201 cells model: couldn't detected any sodium current from cells expressing only the mutant channel, but when it was co-expressed with the WT, the I_{Na} current was recorded at 50% of the normal intensity. This study confirmed that the loss of function of $Na_v1.5$ is the basis for Brugada syndrome[168]. Of course, expression systems have many limitations, but help us to investigate the biophysical properties of mutated ion

[165] *Sendfeld F, Selga E, F S Scornik*, Pérez *G J*, Mills *N L, and* Brugada *R "Experimental Models of Brugada syndrome"* Int J Mol Sci. 2019

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channels involved in cardiomyopathies. This method has been useful also to study LQTs. Indeed, some mutations, like those in potassium channels, are hard to reproduce in mice models, so scientist used expression systems as a first step to understand the consequences of a single mutation in ion channel. January's group used this method to understand the mechanisms behind LQT2 syndrome. LQT2 is associated with delayed repolarization and QT prolongation, often caused by mutation in HERG channel[169] . They tested in HEK cells different mutations of HERG gene and found out that different mechanisms underlie the insurgence of LQT2 symptoms. Mutations Y611H and V822M, for example, cause defects in protein folding and production, hindering the expression of the channel on the cell surface; mutations like I593R and G628S on the same HERG gene, cause alterations that lead to a non-functional channel; again, other mutations could cause damage in the gating properties of the channel[170]; this highlights the variability of the genotype and of the mechanisms behind the same syndrome. In vitro, cell-based disease modelling is not expensive and is easy to maintain and manipulate; in addition, it gives us the chance of studying the consequences of a mutation at single channel level. The limitation of those expression systems is its distance from human cardiomyocytes, in terms of protein expression and the absence of all the extracellular mechanisms involved in regulating channels function. In addition, the response to genetic manipulation and the protein expression is not always the same if different cell lines are used (HEK cells, SV40 Transformed cells [tsA201] or Chinese Hamster Ovary [CHO] cells)[171]

Despite the usefulness of expression systems, also animal models or, even better, human samples, are needed. They could give us a clear representation of the pathology in the heart, at tissue level, and better represent the genetic mechanisms behind the pathology. From invertebrate to vertebrate species (fly, mouse, rabbit and zebrafish), many models have been created to study and classify the different LQT gene mutations[172]. Brugada syndrome has been also designed in different species, from mouse to rabbit[173], the same for hypertrophic cardiomyopathy and dilated cardiomyopathy, which were designed also in big mammals like pigs[174], or studied in

[169] *Curran M. E., Splawski I., Timothy K. W., Vincent G. M., Green E. D., Keating M. T. "A Molecular Basis for Cardiac Arrhythmia: HERG Mutation Cause Long QT Syndrome" Cell 1995*
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[171] *Sendfeld F, Selga E, Scornik FS, Pérez GJ, Mills NL, and Brugada R "Experimental Models of Brugada syndrome"* Int J Mol Sci. *2019*
[172] *Salama G, London B. "Mouse models of long QT syndrome." J Physiol. 2007*
[173] *Sendfeld F, Selga E, Scornik FS, Pérez GJ, Mills NL, Brugada R. "Experimental Models of Brugada syndrome." Int J Mol Sci. 2019*
[174] *Montag J, Petersen B, Flögel AK, et al. "Successful knock-in of Hypertrophic Cardiomyopathy-mutation R723G into the MYH7 gene mimics HCM pathology in pigs." Sci Rep. 2018*

spontaneously-occurring models, like cats for HCM[175] and dogs for DCM[176]. Mouse models are the most common animal model in use, they are not too expensive and easy to take care of. They also have many limitations, starting from the features of the heart tissue: mouse heart beat 10 times faster than the human heart which means shorter action potentials, with huge differences in repolarizing currents (potassium currents). As a consequence, the ECG differs from mouse to human and tachycardia is uncommon in mice as a SCD cause[177]. An evidence of how these differences is important came from the modelling of LQT syndrome; while models of LQT3 and LQT4 exhibit phenotypic outcomes that recapitulate the symptoms seen in human LQT syndrome patients[178] the models for LQT syndrome related to disfunction in potassium current, like LQT 1, 2, 5 and 6, do not represent the pathophysiological features of the human pathology and so are not representative of the pathological condition[179] [180]. Nonetheless, mice are the most common animal models in use. For HCM, the first mouse model carried a missense mutation in the murine α-MyHC gene, corresponding to the human β-MyHC R403Q mutation. This model was used to test the efficacy of L-Type calcium current inhibitor Diltiazem, because it reproduced most of the disease phenotypes. Indeed, those mice showed myocellular disarray, fibrosis, and diastolic dysfunction and the rate of arrhythmogenic events was significantly increased[181]. However, left ventricle (LV) hypertrophy was barely present[182]. A mouse model of "thin filament" HCM have been developed by Tardiff and collaborators using a missense mutation in the cardiac troponin T gene (R92Q); this model showed different hallmarks of the disease such as mitochondrial structural alterations, diastolic dysfunction, hypercontractility and interstitial fibrosis[183]. Isolated cardiomyocytes from this model also shows impaired calcium handling, increased, diastolic calcium concentrations and an increase rate of spontaneous activity. All those features make it an ideal model for the study of HCM and drug discovery[184]. Mouse models have been used to investigate the phenotype of heterozygous *Scn5a* KO mouse and to understand how closely it

[175] *Kittleson, M.D., et al., Familial hypertrophic cardiomyopathy in maine coon cats: an animal model of human disease. Circulation, 1999*
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[182] *Bevilacqua, L.M., et al., QT dispersion in alpha-myosin heavy-chain familial hypertrophic cardiomyopathy mice. Pediatr Res, 1999*
[183] *Coppini, R., et al., Ranolazine Prevents Phenotype Development in a Mouse Model of Hypertrophic Cardiomyopathy. Circ Heart Fail, 2017*
[184] *Tardiff, J.C., et al., Cardiac troponin T mutations result in allele-specific phenotypes in a mouse model for hypertrophic cardiomyopathy. J Clin Invest, 1999*

recapitulated BrS. This model, created by Papadatos and co-workers, recapitulate some features of the pathology but showed also some features that were in contrast with those of BrS in human[185]. Remme and collaborators use a mouse model to study a new mutation in the *SCN5a* gene, responsible for a mixed phenotype between BrS and LQT3: *Scn5a1795insD*[186]. This is a heterozygous model, so it expresses the WT with the mutant channels; mutant mice have a lower heart rate and increased QTc interval and more bradycardia events than WT homozygous mice. Cardiomyocytes isolated from the heart tissue, analysed with the patch clamp technique, displayed a significant reduction of I_{Na} current (without alterations in the activation and inactivation kinetics of the channel) and prolongation of AP duration. This study confirmed that a single mutation in SCN5a gene could be responsible for an overlap syndrome[187]. In conclusion, depending on mutation causing the disease, it will be possible to model the pathology in a mouse model or it may be necessary to employ about different approaches.

Rabbit is another animal model largely used to model hypertrophic cardiomyopathy and channelopathies. It has some features that makes it more similar to the human model, like the heart size, the expression of β-MyHC[188], with cardiac contractile properties similar to those of the human heart[189], and the expression of the human-like K^+ currents, determining a prolonged repolarization of the action potential. The first rabbit model generated for HCM carried the mutation R403Q in the β-MyHC gene[190]. This model perfectly resembles the phenotype of human pathology, including the myocyte disarray and the altered energetic balance; the hypertrophy of the left ventricle was also present as well as fibrosis and diastolic dysfunction. This model was used by Lowery and co-workers to verify the effects of the mutation on the acto-myosin interaction. The study revealed a loss of function in the mutant rabbit, less force is produced by the myofilaments and the kinetics for activation and relaxation were reduced[191] [192]. Transgenic rabbit models have been used to study LQT syndrome and its relationship with arrhythmias and

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[185] *Papadatos GA, Wallerstein PM, Head CE, Ratcliff R, Brady PA, Benndorf K, Saumarez RC, Trezise AE, Huang CL, Vandenberg JI, Colledge WH, Grace AA "Slowed conduction and ventricular tachycardia after targeted disruption of the cardiac sodium channel gene Scn5a." Proc Natl Acad Sci U S A. 2002*

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[191] *Lowey, S., et al., "Hypertrophic cardiomyopathy R403Q mutation in rabbit beta-myosin reduces contractile function at the molecular and myofibrillar levels." Proc Natl Acad Sci U S A, 2018*

[192] *Lowey, S., et al., "Transgenic mouse alpha- and beta-cardiac myosins containing the R403Q mutation show isoform-dependent transient kinetic differences." J Biol Chem, 2013*

SCD[193]. Rabbit expressing the mutant human genes *KCNQ1 and KCNH2* reproduced LQT1 and 2 phenotypes respectively, they were designed by Brunner and collaborators. Those models represented the human features of disease with the prolongation of the action potential duration because of the reduction of I_{Kr} and I_{Ks}. The subsequent studies of optical mapping revealed an increase of the spatial dispersion of the repolarization in the mutant heart tissue, without any compensatory mechanisms, so the presence of a single mutation in HERG or in KvLQT1 protein is enough to cause downregulation of both repolarizing currents and cause arrhythmias, at variance with what was seen in mouse models with the same mutations[194].

All the animal and cellular models have several limitations and of course human samples of heart tissue are the best possible model we could study. Human samples also have some limitations, first of all their availability. In HCM, obtaining samples from patient is pretty common, as septal myectomy is indeed a common surgical option for patients with a hypertrophic heart. Our group performed different works on ventricular cardiomyocytes obtained from surgical reduction of the interventricular septum; we investigated the electrophysiology of cardiomyocytes derived from HCM patients, to better understand the mechanisms behind the pathology, to identify new therapeutic targets and also to test the safety and efficacy of new drugs. From those studies we confirmed the presence of altered Ca^{2+} and Na^{+} homeostasis and, thanks to patch clamp experiments, we also found an increased action potential duration and an increase in arrhythmogenic activity (EAD and DAD), as compared with control samples derived from non-failing/ non hypertrophic patients[195] [196]. In view of these evidences, our group had also tested the efficacy of some specific drugs such as the late sodium channel blockers Ranolazine and GS-967, showing how, prevention of sodium overload has many beneficial effects in HCM hearts[197] [198].

The latest discovery in basic science was the possibility to obtain cardiomyocytes from human induced pluripotent stem cells (hiPSC). Since Yamanaka and Takahashi report the reprogramming protocol[199] of terminally differentiated cells into the embryonic-state-like of induced pluripotent stem cells (iPSC), many ethical concerns have been overpassed and the fetal like cells have become largely used. Human iPSC gives scientists the possibility to obtain human

[193] *Ziv O, Morales E, Song YK, et al. "Origin of complex behaviour of spatially discordant alternans in a transgenic rabbit model of type 2 long QT syndrome." J Physiol. 2009*
[194] *Brunner M, Peng X, Liu GX, et al. "Mechanisms of cardiac arrhythmias and sudden death in transgenic rabbits with long QT syndrome." J Clin Invest. 2008*
[195] *Coppini R, Ferrantini C, Pioner JM, et al. Electrophysiological and Contractile Effects of Disopyramide in Patients with Obstructive Hypertrophic Cardiomyopathy: A Translational Study. JACC Basic Transl Sci. 2019*
[196] *Coppini, R., et al., Altered Ca (2+) and Na (+) Homeostasis in Human Hypertrophic Cardiomyopathy: Implications for Arrhythmogenesis. Front Physiol, 2018*
[197] *Coppini, R., et al., Late sodium current inhibition reverses electromechanical dysfunction in human hypertrophic cardiomyopathy. Circulation, 2013*
[198] *Ferrantini, C., et al., "Late sodium current inhibitors to treat exercise-induced obstruction in hypertrophic cardiomyopathy: an in vitro study in human myocardium." Br J Pharmacol, 2018*
[199] *Takahashi K, Yamanaka S. "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell. 2006*

fetal like cells of almost all human tissues, including the cardiac, without embryos disruption and maintaining the genetic background of the donor patient. This technique of reprogramming somatic cells to an embryonic phenotype and then differentiate it in another phenotype, different from the first one, give the scientists the opportunity of having a large source of differentiated cells of many tissues, including those difficult to obtain from surgical pieces or impossible to grow up in culture. The Yamanaka factors are fundamental for reprogramming the somatic cells and then, in our case, the differentiation of the hiPSC in cardiomyocytes occur thanks to different factors added in culture at specific time points to mimic the embryonic development of the heart tissue. Those cells, as obtained from human terminally differentiated cells of a patient, are actually human and have all the genetic pool of the patient, even mutations, if they are present in the genome of whom they derived from. The chance of keeping unaltered the genetic background of the patients and obtain a cellular model of cardiomyocytes to grow up in culture, give researchers the opportunity to investigate the development of the heart tissue in vitro if an healthy donor is the source of somatic cells or to better understand the pathogenic mechanisms behind a cardiac genetic determined disease, when a patient affected from a genetic cardiac disease is used as source of somatic cells to obtain hiPSC; in that way researchers are allowed to study all the mechanisms altered by specific mutations related to cardiac disease and their occurrence during cardiac development. Liang, Wu and co-workers created an hiPSC line starting from skin fibroblast of BrS patients and then differentiate cardiomyocytes to study the mechanisms of the disease and to verify the reliability of the model. With genome editing techniques, they also generate an isogenic control line. They found, in mutant cardiomyocytes, all the hallmarks of the disease including abnormalities in Ca^{2+} handling, reduced peak sodium current and increased triggered activity. So, their model is reliable and useful for drug screening and to get insight on the pathology[200]. Veerman and collaborators also used cardiomyocytes derived from hiPSC to investigate Brugada syndrome in patients where the mutation causing the disease was unknown. Studying the electrophysiological properties of hiPSC derived cardiomyocytes they discovered no differences in up stroke velocity and peak sodium current between control and patient derived cardiomyocytes. L-type calcium current and transient outward potassium current were also unaltered. These evidences from a Brugada syndrome patient shows that mutations in SCN5a channel is not a standard in BrS[201]. To better understand the complicated pathomechanisms behind HCM phenotype, Lan's group generated hiPSC lines from ten members of a family affected by a mutation in the MYH7 gene causing HCM. They performed calcium imaging on those cardiomyocytes and found out, in the cellular model, the same hallmarks that are present in

[200] *Liang P, Sallam K, Wu H, et al. "Patient-Specific and Genome-Edited Induced Pluripotent Stem Cell-Derived Cardiomyocytes Elucidate Single-Cell Phenotype of Brugada Syndrome." J Am Coll Cardiol. 2016*

[201] *Veerman, C., Mengarelli, I., Guan, K. et al. hiPSC-derived cardiomyocytes from Brugada Syndrome patients without identified mutations do not exhibit clear cellular electrophysiological abnormalities. Sci Rep 2016*

human cardiomyocytes: abnormal calcium cycling and calcium overload. Then, they used it as a representative model for drug screening and to better understand the role of calcium in hypertrophic remodelling[202]. hiPSC cardiomyocytes have been generated also starting from patients with dilated cardiomyopathy. In 2012 Sun and collaborators create a DCM line from a family carrying a mutation (R173W) in the cardiac troponin T gene. Those cardiomyocytes exhibited a decreased contractility, abnormal calcium handling and also abnormal distribution of alpha actinin, reproducing the principal features of DCM including abnormal response to β-adrenergic stimulation and improvement after treatment with β-blockers[203]. The first hiPSC line used to study LQT syndrome was derived from dermal fibroblast of two family members with a non-sense mutation on the KCNQ1 gene, encoding for a potassium channel responsible for I_{Ks} and causing LQT1 syndrome. The study revealed that this mutation causes a reduction in I_{Ks} currents because of trafficking defects and alteration in activation-inactivation kinetics. This model also shows improvement in reducing arrhythmic events after β-blockers treatment, like human cardiomyocytes. So, the culture is able to resemble the main features of LQT1 syndrome[204]. As described, the possibility to derived cardiomyocytes from somatic cells of patients is an important tool to study cardiomyopathies. Those cells perfectly resemble the genetic pool of the patients they are derived from, but, at the same time, culture could be easily genetically manipulated to obtained isogenic control lines or to induce the expression of specific mutations[205]. They are a safe and reliable source of human cardiomyocytes. On the other hand, they're not easy to keep in culture and their maturation is usually not optimal. Indeed, these cells are fetal-like cardiomyocytes, and they do not reach fully adult phenotype[206]. this means that there are many differences in ion currents and action potential shape as compared with control adult cardiomyocytes[207]. Specific protocols to induce the differentiation in a specific cardiomyocyte,

[202] *Lan F, Lee AS, Liang P, et al. "Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells." Cell Stem Cell. 2013*
[203] *Sun N, Yazawa M, Liu J, et al. Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. Sci Transl Med. 2012*
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[207] *Casini S, Verkerk AO, Remme CA. Human iPSC-Derived "Cardiomyocytes for Investigation of Disease Mechanisms and Therapeutic Strategies in Inherited Arrhythmia Syndromes: Strengths and Limitations." Cardiovasc Drugs Ther. 2017*

ventricular, atrial or nodal like, have also been designed but is not easy to obtained a pure culture[208] [209] [210].

[208] *Devalla HD, Schwach V, Ford JW, Milnes JT, El-Haou S, Jackson C, et al. "Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology." EMBO molecular medicine. 2015*

[209] *Protze SI, Liu J, Nussinovitch U, Ohana L, Backx PH, Gepstein L, et al. "Sinoatrial node cardiomyocytes derived from human pluripotent cells function as a biological pacemaker." Nat Biotechnol. 2017*

[210] *Lian X, Hsiao C, Wilson G, Zhu K, Hazeltine LB, Azarin SM, et al. "Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signalling." Proc Natl Acad Sci U S A. 2012*

AIM

Modelling cardiomyopathies has always been challenging for basic science studies because of the large variability in the genetic background and in the phenotype manifestations. Common features of all cardiomyopathies are the cardiac dysfunction and the high predisposition to arrhythmic events that could lead to sudden death. The identification of an appropriate drug treatment to reduce the incidence of arrhythmic events or restore the cardiac function, is crucial for affected patients. Considering the variety of causes and potential pathogenic mechanisms leading to lethal arrhythmias associated to cardiomyopathies, it is clear that there could not be a single approach to model this large group of heart muscle dysfunctions. Different models are useful to gain deeper insight into the pathogenic mechanisms underpinning the disease and will help us to identify an appropriate pharmacological treatment. For this reason, I established to use different approaches to model different types of cardiomyopathies; in particular, I will use "expression systems" to investigate a mixed phenotype between BrS and LQT3, caused by the 1795insD mutation on *SCN5A* gene. Then, I will investigate the effects of Disopyramide on obstructive HCM using cardiomyocytes isolated from human surgical samples; finally, I also used hiPSC derived cardiomyocytes to model DCM associated to the Duchenne muscular dystrophy, an inherited disease. I would confirm the crucial role of the aforementioned approaches in basic science, in order to evaluate the more suitable technique to model a specific disease and to screen the efficacy of old and novel drugs in the presence of certain mutations.

METHODS

In this thesis I will describe different methods to study cardiomyopathies and ion channel defects in the heart; we will investigate the mechanical and electrophysiological properties of cardiomyocytes and kinetics properties of ion channel. We used expression systems to investigate cardiac sodium channel defects and cardiomyocytes derived from human tissue samples or hiPSC to investigate the feature of two models of cardiac dysfunction, the diastolic dysfunction, typical of the hypertrophic cardiomyopathy and the systolic dysfunction present in dilated cardiomyopathy; for the first one we will use human samples, for DCM, due to the difficult in obtain cardiac samples, we used cardiomyocytes derived from hiPSC. We mostly used electrophysiological techniques, patch clamp and calcium transient measurements using calcium fluorescent probes, to analyse the electrophysiological properties of those models and their alterations related to the diseases.

The SCN5A1795insD mutation: an overlap syndrome

The human cardiac sodium channel Nav 1.5, responsible for the rapid sodium influx that determine the upstroke of the action potential and the membrane depolarization, is susceptible to several mutations, many of which are associated with life-treating arrhythmias and sudden cardiac death. The gene called sodium voltage-gated channel alpha subunits 5, *SCN5A,* encode for the alpha subunit of Nav 1.5 channel and is located on chromosome 3. The Nav channel is a multicomplex protein composed by regulatory beta-subunits and an ion conducting alpha-subunit. The latest is the pore forming subunit of the channel, it has four domains, each composed by six transmembrane segments; the segment 4 of each domain make the voltage sensing domain[211]. Here we will analyse a specific mutation of *SCN5A*, discovered for the first time in a large Dutch family affected by a mixed phenotype between BrS and LQT3; among the family members there was a high incidence of nocturnal SCD, the ECG showed the typical features of Brugada syndrome and the prolongation of QT-interval. This was a mixed phenotype between LQT3 and BrS[212]. The gene mutation found in this family cause the insertion of an aspartate after tyrosine 1795 (1795insD) at the C- terminal domain of the α-subunit of Nav 1.5 channel. Studying the electrophysiological properties of the mutant channel, Bezzina and co-workers discovered that the peak sodium current intensity was significantly reduced, but at the same time, the mutation

[211] *Gellens ME, George AL Jr, Chen LQ, et al. "Primary structure and functional expression of the human cardiac tetrodotoxin-insensitive voltage-dependent sodium channel." Proc Natl Acad Sci U S A. 1992*

[212] *Bezzina C, Veldkamp MW, van Den Berg MP, et al. A single Na (+) channel mutation causing both long-QT and Brugada syndromes. Circ Res. 1999*

altered the gating properties of the channel causing a sustained sodium current (late sodium current) during the action potential plateau, therefore prolonging the repolarization phase[213].

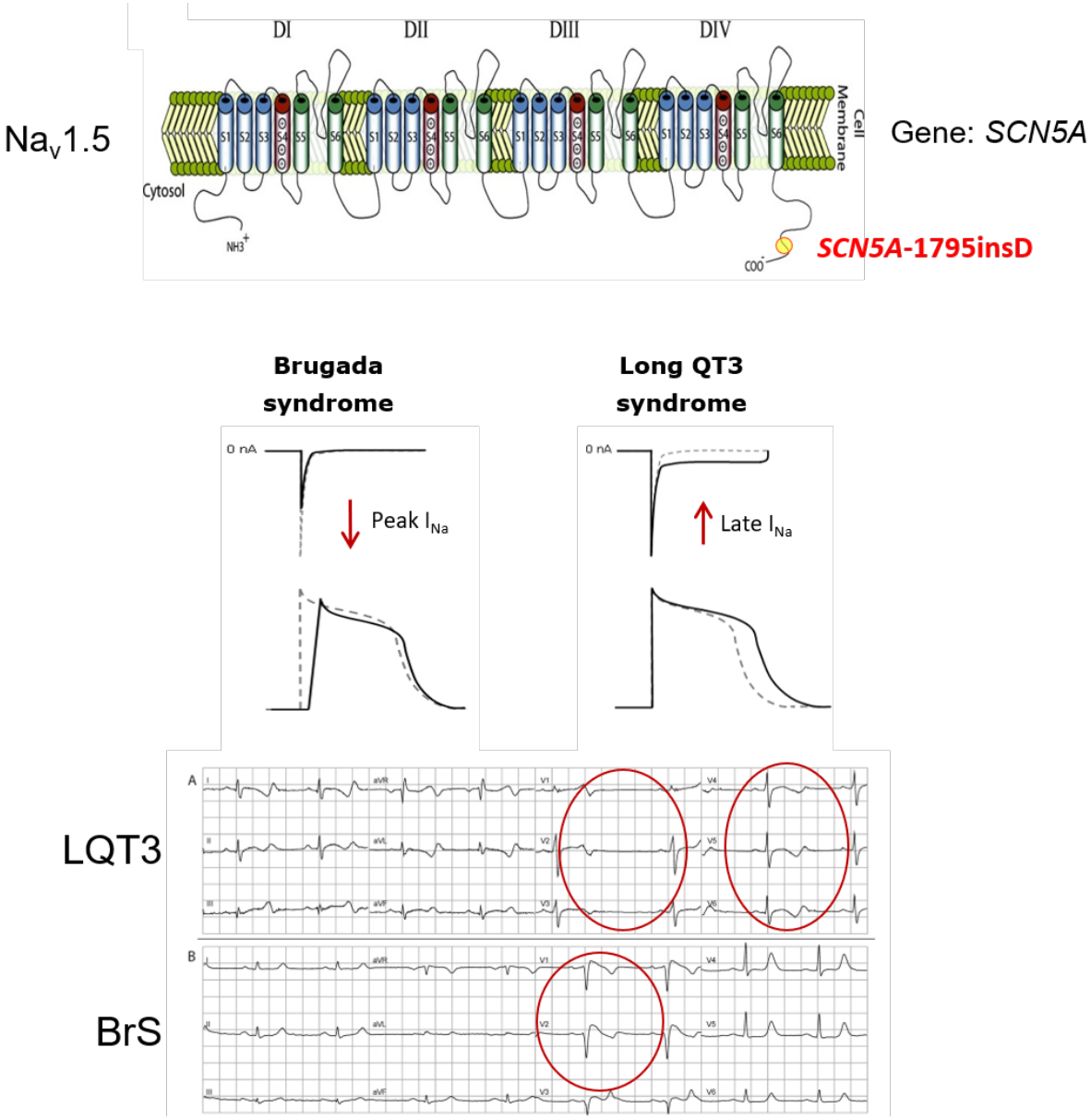

Figure 7: Cardiac sodium channel and the mixed phenotype

On the top, the structure of cardiac sodium channel Nav 1.5 encode by SCN5A gene, made up by 4 transmembrane domains (DI, DII, DIII, DIV), each one with six transmembrane segments (S1 to S6), of those, the segments 4 are the voltage sensors. The mutation of our interest, 1795insD, is located in the C-terminal region. In the middle: the alterations of the cardiac sodium current associated with BrS or LQT3S; the loss of function mutation associated with Brugada syndrome cause a reduction of peak sodium current causing a slowing of the rapid upstroke of the action potential; the gain of function mutation associated with LQT3 syndrome cause an increment of the Late sodium current responsible for a prolongation of the plateau phase of the action potential.

[213] *Guzik TJ, West NE, Black E, et al. "UltraRapid communications: vascular superoxide zproduction by NAD(P)H Oxidase Association with endothelial dysfunction and clinical risk factors." Circ Res. 2000*

The phenotype associated with the SCN5A mutation 1795insD is a mix between those two. In the end we can see the abnormalities in the ECG registration associated with the mixed phenotype.

The 1795insD mutation on *SCN5A* gene, has been studied in different models, from animals to hiPSC. After many studies in vitro to characterize the electrophysiological properties of the mutant channel using HEK293 cells or xenopus oocytes, an in vivo model was necessary to better understand the consequences of the mutation in its original context. The first was a mouse model carrying the equivalent of the human mutation in mice; heterozygote mouse model perfect resembled the human phenotype, including bradycardia, QT prolongation and right ventricle conduction slowing[214]. Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CM) have been used to study the mutation in human cardiomyocytes and to test drug efficacy; Portero, Casini and co-workers test the efficacy of a selective inhibitor of I_{NaL}, GS967, on mutant sodium channels in an hiPSC-CM model. Compared to cardiomyocytes isolated from mutant mice, 1795insD hiPSC-CM resembled the principal mutant channel kinetics features. Exposure to GS 967 reduced I_{NaL} and the duration of the action potential without changing the amplitude of peak sodium current in all the cardiomyocytes tested (isolated from mutant mice and derived from hiPSC)[215]. This is the proof that hiPSC-CMs are a valuable tool to obtain a human in vitro model of ion channel diseases reliable as the animal model.

Mexiletine: a possible way to restore a mixed phenotype

The 1795insD mutation in the cardiac sodium channel causes an ion channel disease that is currently without a specific pharmacological treatment. Scientists are looking for a molecule that is able to reduce the late sodium current but at the same time increases the reduced peak sodium current in the mutant Nav1.5, without altering the WT channel function. Mexiletine is a small molecule used as antiarrhythmic agent (class Ib) and local anaesthetic; it inhibits the sodium current reducing the up-stroke velocity of the action potential[216]. Class Ib antiarrhythmic drugs target the voltage-sensitive cardiac sodium channel, mostly decreasing the late sodium current, shortening the action potential duration and prolonging the refractory period[217]. Administration of those drugs is not always safe[218]. Zhu and collaborators supposed that the efficacy of

[214] *Remme CA, Verkerk AO, Nuyens D, et al. Overlap syndrome of cardiac sodium channel disease in mice carrying the equivalent mutation of human SCN5A-1795insD. Circulation. 2006*

[215] *Portero V, Casini S, Hoekstra M, et al. Anti-arrhythmic potential of the late sodium current inhibitor GS-458967 in murine Scn5a-1798insD+/- and human SCN5A-1795insD+/- iPSC-derived cardiomyocytes. Cardiovasc Res. 2017*

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[217] *Zipes DP, Jalife J. "Cardiac Electrophysiology: From Cell to Bedside: Sixth Edition." 2013*

[218] *Echt DS, Liebson PR, Mitchell LB, et al. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The Cardiac Arrhythmia Suppression Trial. N Engl J Med. 1991*

Mexiletine as sodium channel blockers is dependent on the mutation affecting the channel because it binds (like Lidocaine) the pore forming domain of the channel that is connected with the voltage-sensing-domain. Mutations that alter the coupling mechanisms that allows the channel to change conformation after a changing of membrane voltage, result in different response to Mexiletine treatment. Anyway, Mexiletine seems to block I_{Na} by a different mechanism than Lidocaine; indeed, it stabilizes the inactivated conformation of the channel without shifting the steady state inactivation curve (like Lidocaine does)[219]. Sodium channel blockers are in use to treat Long QT syndrome type 3, associated to *SCN5A* mutation, but, as already said, their efficacy depends on the specific mutation on the *SCN5A* gene. A study from 2010 provided evidence that, in the presence of specific mutations, Mexiletine could be useless to restore QT duration and could even be dangerous, because it caused a QT prolongation by facilitating the trafficking of the mutant channel to the cell surface[220]. In 2004, Valdivia and its group identified a mutation in *SCN5A* responsible for BrS, causing retention of the mutant channel in the endoplasmic reticulum. Expressing this mutant channel (G1743R) in HEK293 cells, they studied the trafficking and the response to class Ib antiarrhythmic drugs (Mexiletine and Quinidine). G1743R *SCN5A* mutant channel has an undetectable peak sodium current, the treatment with Mexiletine partially restored the peak current in HEK293 cells exposed for 48h at 500μM of drug. Using a FLAG to tag the mutant channel and immunohistochemistry analysis, they showed how the absence of peak current is due to the retention of the channel in the ER and the administration of Mexiletine actually promoted the expression of the channel in the cell membrane[221]. Many works investigated the ability of mexiletine to restore protein trafficking from the endoplasmic reticulum to the membrane, but the mechanism behind this capability still remains unclear, as are the dose-dependence efficacy and its mutation specificity[222] [223] [224]. All this unknown and unpredictable effects of mexiletine make it difficult to manage it but its capability to act as a late sodium current inhibitor and as an enhancer of peak sodium current by rescuing the channel trafficking, make it a good contender to be a specific treatment in mixed phenotype pathology involving cardiac sodium channel dysfunction.

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[219] *Zhu W, Mazzanti A, Voelker TL, et al. Predicting Patient Response to the Antiarrhythmic Mexiletine Based on Genetic Variation. Circ Res. 2019*

[220] *Ruan Y, Denegri M, Liu N, et al. "Trafficking defects and gating abnormalities of a novel SCN5A mutation question gene-specific therapy in long QT syndrome type 3." Circ Res. 2010*

[221] *Valdivia CR, Tester DJ, Rok BA, et al. "A trafficking defective, Brugada syndrome-causing SCN5A mutation rescued by drugs." Cardiovasc Res. 2004*

[222] *Valdivia CR, Ackerman MJ, Tester DJ, Wada T, McCormack J, Ye B, Makielski JC. A novel SCN5A arrhythmia mutation, M1766L, with expression defect rescued by mexiletine. Cardiovasc Res. 2002*

[223] *Tan BH, Valdivia CR, Song C, Makielski JC. Partial expression defect for the SCN5A missense mutation G1406R depends on splice variant background Q1077 and rescue by mexiletine. Am J Physiol Heart Circ Physiol. 2006*

[224] *Ruan Y, Denegri M, Liu N, et al. "Trafficking defects and gating abnormalities of a novel SCN5A mutation question gene-specific therapy in long QT syndrome type 3." Circ Res. 2010*

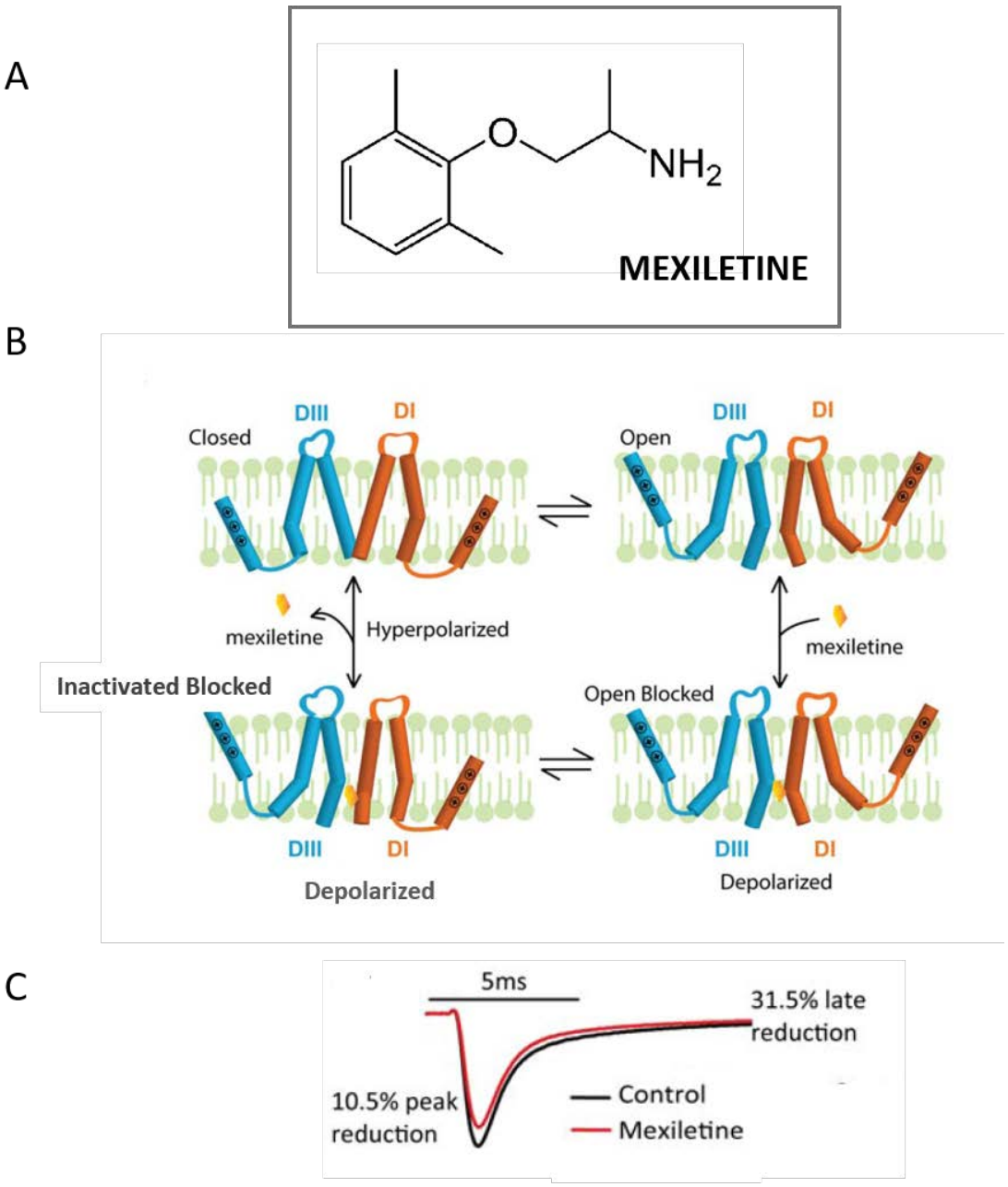

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Figure 8: Mexiletine

A schematic representation of Mexiletine, a sodium channel blockers class Ib followed by a representation of its interaction with the channel. As described in the work of Zhu in Circulation, Mexiletine binds the domain I and III when the channel is in the open state and it block the current; Mexiletine leave the channel when membrane is hyperpolarized and the channel closes its gate. The entity of the sodium channel block is represented in section C. Mexiletine reduces the peak current of about 10.5% and 31.5% of the late sodium current, accordingly with the work of Zhu, after the acute exposure to 250 μM/L of Mexiletine[225].

[225] *Zhu W, Mazzanti A, Voelker TL, Hou P, Moreno JD, Angsutararux P, Naegle KM, Priori SG, Silva JR. Predicting Patient Response to the Antiarrhythmic Mexiletine Based on Genetic Variation. Circ Res. 2019*

Heterologous expression and drug treatment

To study the effect of Mexiletine in *SCN5A* 1795insD mutant sodium channel we decided to use Human Embryonic Kidney Cell line (HEK293) expression system, to analysed the direct effect of the molecule on the channel and characterized the kinetics alterations.

cDNA for Na^+ channel was prepared by mutagenesis starting from a double strand pSP64T-hH1(sp) plasmid of *SCN5A*. then the cDNA was subcloned with the pCGI vector to have the expression of both, channel protein and GFP reporter. HEK293 cells were transiently co-transfected with 400ng of WT- or 1795insD-SCN5A cDNA ($Na_v1.5$ α-subunit) and 400ng $h\beta_1$ regulatory subunit (SCN1B) c-DNA using Lipofectamine. After 6/8 hours of incubation with transfection medium we change it with a minimum essential medium for culture, added of mexiletine 500μM or vehicle and incubated overnight or 48 hours before performing the patch clamp analysis.

Colture Medium: **DMEM** (GIBCO 21969) added with 10% **FCS**, 1% **P\S**, 1% **L. Glut**.

Transfection Medium: **DMEM** (GIBCO 21969) added with 1% **P\S** and 1% **L. Glut**.

HCM studies and Disopyramide treatment for obstructive patients

HCM, as previously mentioned, is a heterogeneous monogenic heart disease associated in many cases with arrhythmias, heart failure and even sudden death, consisting in a complete remodelling of the heart tissue that leads to the thickening of the left ventricle walls, especially of the interventricular septum. The increase interventricular septum thickness could be so serious to determine the left ventricle outflow tract obstruction and make, for those patients, priority for myectomy operation to relieve the obstruction. Surgery is needed when the pharmacological treatment in use, that are usually not specific (β-adrenergic antagonist or calcium channel blockers like Verapamile), fail in restoring symptoms (dyspnoea, syncope and atrial fibrillation). Disopyramide is an antiarrhythmic drug (Class IA), it has a negative inotropic and dromotropic effects due to its anticholinergic activity that inhibits parasympathetic tone[226]. As a class I antiarrhythmic drug, it acts as a sodium channel blocker lengthening the action potential duration, reducing the up-stroke velocity so shifting to the right the action potential curve. Disopyramide is also able to avoid arrhythmic events in cells with an increase spontaneous activity, slowing the depolarization phase of AP. Disopyramide, like other Ia antiarrhythmic drugs, also acts on delayed rectified potassium current, blocking the rapid component of this current, thus resulting,

[226] *January CT, Wann LS, Alpert JS, et al. 2014 AHA/ACC/HRS guideline for the management of patients with atrial fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and the Heart Rhythm Society [published correction appears in J Am Coll Cardiol. 2014*

together with the block of I_{Na}, in QT interval prolongation. Due to its anticholinergic properties, its ability to block sodium channel and inhibit the delayed rectified potassium current, side effects are pronounced. It's ability to prolong the QT interval make it dangerous for all those syndromes associated to a prolonged QT tract, and after myocardial infarction to avoid arrhythmic events; it should be administered with caution also in elderly patients. Today Disopyramide is rarely used to treat heart rhythm abnormalities because of its side effects but it is still the drug of choice for arrhythmias vagally mediated. Disopyramide is also use in HCM patients with LVOT obstruction because, thanks to its negative inotropic effect, is able to reduce the LV contractility and slowdown the acceleration of flow in the outflow tract during diastole; even in those cases its use is limited by its side effects as an anticholinergic drug[227]. Even if Disopyramide is in use since 1980, no much work has been done to investigate its mechanism of action at molecular level, and we still don't know if it has an action on calcium homeostasis or on calcium release from SR, not even if it interacts with the actin-myosin coupling[228].

Isolation of cells from human cardiac samples

We tested the effects of Disopyramide on cardiac human samples derived from patients who goes under myectomy to reduce the septal thickening and LVOT obstruction. Human septal specimens from HCM and control surgical patients were collected in cold cardioplegic solution containing (in mmol/L): KH_2PO_4 50, $MgSO_4$ 8, HEPES 10, adenosine 5, glucose 140, mannitol 100, taurine 10 (pH 7.4 with KOH). Tissue was in part rapidly frozen in liquid Nitrogen, in part processed to isolate responsive single cells or trabeculae. To obtain single cells for patch clamp analysis, the tissue sample is minced to small pieces (~$1mm^3$) and subjected to enzymatic and mechanical dissociation. we used a scraping custom-made device and Ca^{2+}-free bathing solution, the Isenberg solution, containing in mM: NaCl 120, KCl 10, KH_2PO_4 1.2, $MgCl_2$ 1.2, Glucose 10, Taurina 20, Pyruvate 5, Hepes 10, ph 7.2 adjust with NaOH and added Collagenase Type V and Protease Type XXIV (Sigma) while constantly keep in the solution at 37°C. We were able to digest mechanically and enzymatically the cardiac tissue samples and obtained single cardiomyocytes after 2 hours. During digestion, the buffer containing dissociated myocytes was collected every 15 minutes from the scraping device and diluted with KB solution at room temperature. KB solution contained (in mM): KCl 20, KH_2PO4 10, glucose 25, mannitol 5, L-glutamic acid monopotassium salt 70, β-hydroxybutyric acid 10, EGTA 10 and 2mg/mL albumin (pH 7.2 with KOH) and has the function to inhibit the enzymes' activity. The myocytes were left to settle and

[227] *Ferrantini C, Pioner JM, Mazzoni L, et al. Late sodium current inhibitors to treat exercise-induced obstruction in hypertrophic cardiomyopathy: an in vitro study in human myocardium. Br J Pharmacol. 2018;*
[228] *Coppini R, Ferrantini C, Pioner JM, et al. Electrophysiological and Contractile Effects of Disopyramide in Patients with Obstructive Hypertrophic Cardiomyopathy: A Translational Study. JACC Basic Transl Sci. 2019*

then resuspended in Ca^{2+}-free Tyrode solution containing (in mM): 132 NaCl, 4 KCl, 1.2 $MgCl_2$ 10 HEPES, and 11 glucose (pH 7.35 NaOH). After digestion cardiomyocytes need to be readapted from a Ca^{2+}-free solution to a final concentration of 0.6mM of calcium. To reach this concentration $CaCl_2$ was added stepwise. Cells were stored in this solution and used within 3 hours.

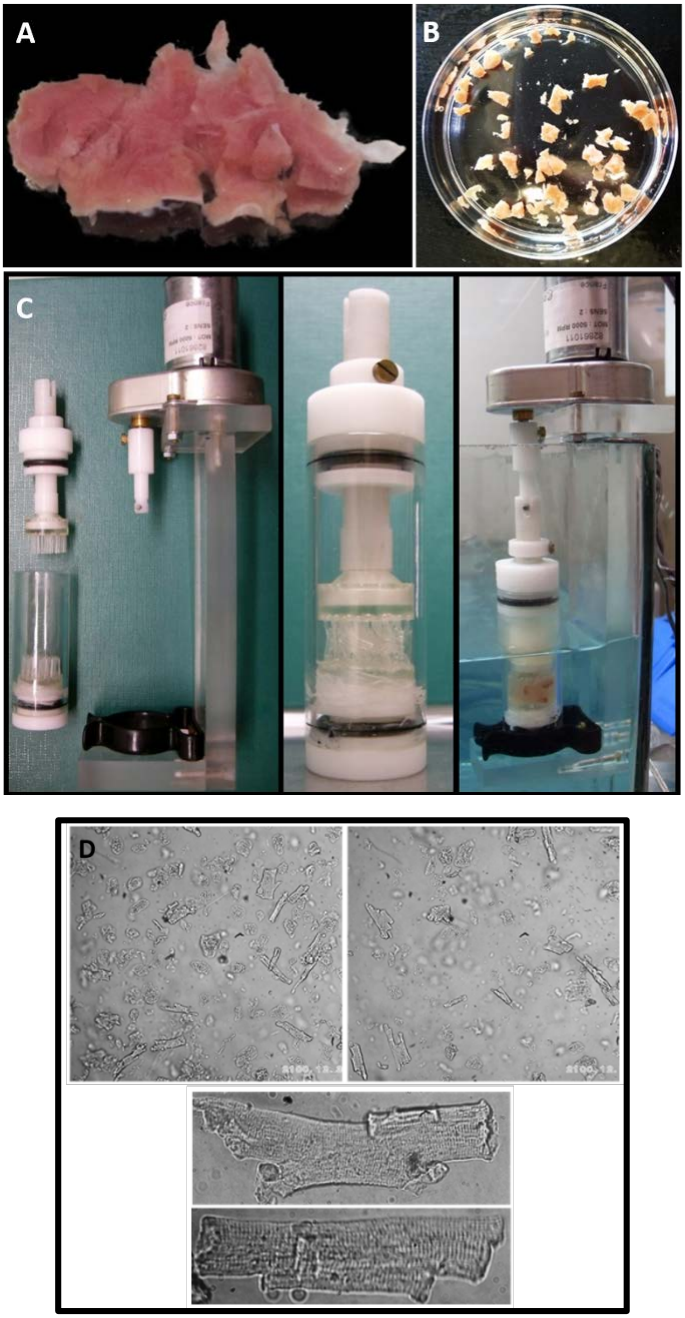


Figure 9: ***Human cardiomyocytes isolation***

A*. Cardiac tissue samples obtained from an HCM patient after surgery to reduced septum hypertrophy;* ***B****. Cardiac tissue minced in small pieces before starting the enzymatic and mechanic digestion.* ***C****. Scraping custom made device to digest the sample and obtain single cardiomyocytes; D. Microscopic view (20x) of cardiomyocytes freshly isolated from the sample.*

Electrophysiological registrations

We performed patch clamp registrations in HEK293 cells to measure the sodium current intensity and in cardiomyocytes isolated from human cardiac samples to measure action potential duration, calcium, sodium and potassium current. Sodium currents (I_{Na}) in HEK293 cells were measured in whole cell voltage clamp configuration, at room temperature (20°C) with the use of an Axopatch 200B amplifier (Axon instruments). Cells were selected on the base of fluorescence and electrode resistance were around 2-3 MΩ. The protocols used for each recording are all described in the figures. In all Na-current protocols, a holding potential of −120 mV and a cycle time of 5 s were used. Currents were filtered at 5 kHz and digitized at 20 kHz. For sodium current recordings, the bath solution contained: NaCl 140mM, CsCl 10mM, $CaCl_2$ 2mM, $MgCl_2$ 1mM, Glucose 5mM, Hepes 10mM, pH 7.4 with NaOH; the pipet solution contained: CsF 110mM**,** CsCl 10mM, NaF10 mM, $MgCl_2$ 1mM, $CaCl_2$ 1mM, Na_2ATP 2mM, EGTA 11mM, hepes 10mM, pH 7.2 CsOH.

To measure calcium current and membrane potential in human isolated cardiomyocytes, perforated patch, whole-cell voltage clamp and current-clamp was performed, in all the experiments the solution was kept at 35±5 °C.

For perforated patch clamp experiments, we used the amphotericin method (Rae J et al. 1991). For action potential recordings, the pipette solution contained (in mM) 115 KMES (potassium methanesulfonate), 25 KCl, 10 HEPES, 3 $MgCl_2$. The standard Tyrode bath solution contained (in mmol/L) 136 NaCl, 5.4 KCl, 0.33 Na_2PO_4, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 dextrose, and 10 HEPES-NaOH; pH was adjusted to 7.35 with NaOH. Membrane potential and action potentials were measured after short stimuli of 3ms at different stimulation frequencies (1-3-5 Hz); in order to reach the steady state, before recording, the stimulation at the chosen frequencies is maintained for at least 1min. To measure the occurrence of arrhythmic events (DADs and EADs) we recorded action potential generation during a pause from stimuli of 20sec following 30 sec of 5Hz stimulation. Membrane potential signals were measured with a Multiclamp 700B (Molecular Devices) patch-clamp amplifier and were filtered at 2 KHz and digitized through a 16-bit A/D converter (Digidata 1440A, sampling rate 5 KHz; Molecular Devices, Inc.). For I_{CaL} recordings, the pipette solution contained (in mM) 80 CsMES (Cesium methanesulfonate), 40 CsCl, 10 HEPES, 1 KCl, 1 CaCl2, pH 7.4 (CsOH) and the bath solution contained (in mM) 140 NaCl, 6 CsCl, 10 glucose, 10 HEPES, 1 MgCl2, 2 CaCl2, pH 7.35 (with CsOH).

Trace acquisition and analysis was controlled by dedicated software (pClamp 10; Molecular Devices).

Calcium imaging

To measure calcium concentration and fluxes between cytosol and sarcoplasmic reticulum in human single cardiomyocytes, we used fluorescent calcium probes. These fluorescents molecules are able to go through the cell membrane, binding Ca^{2+}; then, when excited at a specific wavelength, they are able to emit light at a different wavelength that will be captured by a camera. We associated an inverted microscope to a LED source of light and high-speed cameras (high-speed high-sensitivity EMCCD Camera, model Evolve Delta by Photometrics, USA Photometrics Cascade 128+, Photometrics Evolve Delta). Isolated myocytes were loaded by incubating cells for 30 minutes in Tyrode bath solution containing 10 μmol/L of dye, Cal530 (Quest Bioscience), together with Pluronic buffer to facilitate probe entrance into the cells. After 25 min, cells are left to settle for 5 min and then, when incubation time ends, washed two times with Tyrode solution. A small amount of myocyte suspension (0.3 ml) is put in the microscope chamber where temperature is checked and set at 35±5 °C and the field stimulation is possible thanks to two platinum electrodes on the sides of the chamber. After waiting 3\4 min to let myocytes adhere to the glass bottom of the chamber, we started the perfusion system that helps us to renew constantly (0,3 ml/min) the bath solution, and eventually also allows us to change the chamber solution with those containing the drugs we want to test. The perfusion solution is kept at controlled temperature around 38±5 °C thanks to a heating system (Warner). Cardiomyocytes suspension washed with Tyrode solution, is used for 30 minutes after incubation with the dye. The emission and excitation wavelengths of the calcium probe (CAL520) are 490nm (EX)/520nm (EM)

We recorded the fluorescence emitted at 520nm wavelength by the calcium probes after excitation at 490nm with a LED monochrome light source, thanks to a fast acquisition camera (Photometrics Cascade 128+, Photometrics Evolve Delta). To obtain calcium transients, cell suspension is stimulated at different frequencies (1-3-5 Hz) with square pulses of less than 3 msec. Measurements were performed at steady state by averaging 5-10 subsequent Ca^{2+} transients recorded at steady state.

In order to quantify the occurrence of arrhythmic events, cells were paced at 5Hz for at least 30 seconds and then stimulation was abruptly stopped for 15s, while recording intracellular Ca^{2+} movements. We quantified the occurrence of Ca^{2+} waves (slow raise kinetics, amplitude 20-50% of a regular transient) and spontaneous Ca^{2+} transients (fast upstroke, amplitude comparable to a regular transient). The protocol was repeated in the presence of Isoproterenol (0.1μM).

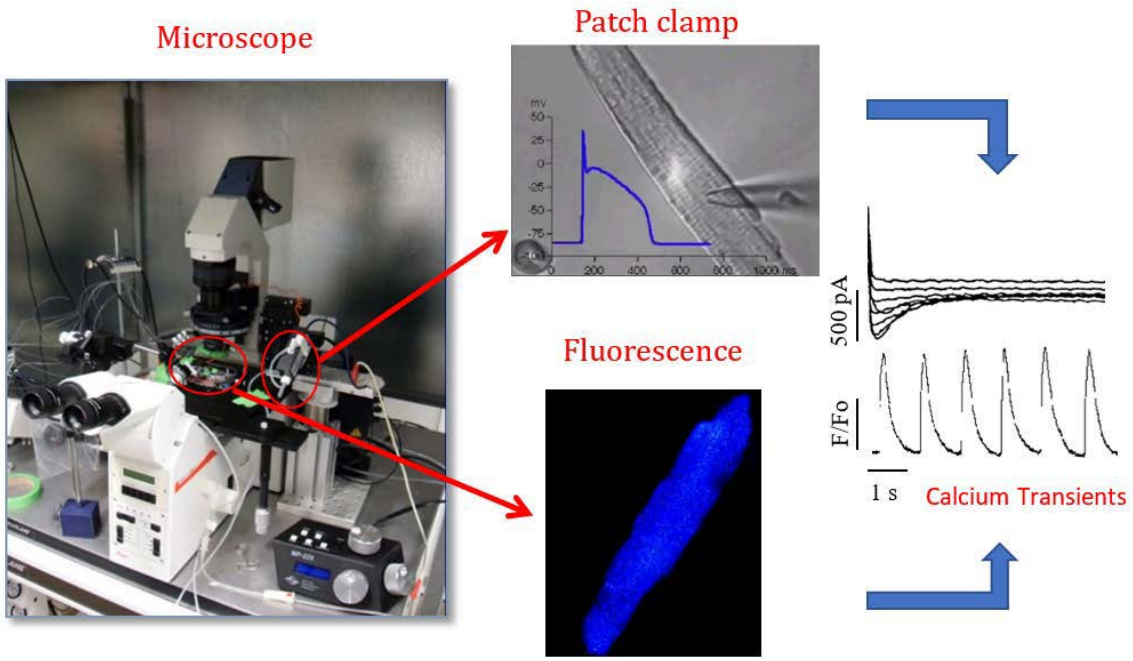


Figure 10. Equipment for patch clamp and calcium transient registration

Our equipment made of an inverted microscope (Leica DMi8), a fast acquisition camera (Photometrics Cascade 128+, Photometrics Evolve Delta) and a LED monochrome lamp; a micromanipulator (Wagner) for patch clamp registration, associated to a Multiclamp 700B (Molecular Devices) patch clamp amplifier and a digitizer (Digidata 1440A, Molecular Devices)

Modelling dilated cardiomyopathy associated with systolic dysfunction with hiPSC

It is known that Duchenne muscular dystrophy (DMD), despite it was consider predominantly as a skeletal muscle disease, it also involves the muscular heart tissue in later stage of the disease, causing dilated cardiomyopathy. DMD is caused by mutations in the dystrophin gene that lead to a truncated or non-functional protein. Dystrophin exerts both functional and structural roles, it is part of the dystrophin-associated glycoprotein complex that links the cytoskeleton to the extracellular matrix. The absence of dystrophin increases cells vulnerability making them more susceptible to the mechanical stress of contraction. Cardiac disease evolves slowly in DMD patient, usually starting with the hypertrophy of cardiomyocytes, suddenly followed by atrophy and fibrosis[229]. Nowadays, there are no specific treatments for these patients, to treat skeletal muscle impairment or the related DCM. In order to ameliorate the quality of life of these patients and to prolong their survival, it is important to better investigate all the DMD related abnormalities, including those of the heart tissue which are becoming the principal cause of death in these patients. It is difficult to obtain cardiac samples from surgery in patients with DMD, and

[229] *Judge DP, Kass DA, Thompson WR, Wagner KR. Pathophysiology and therapy of cardiac dysfunction in Duchenne muscular dystrophy. Am J Cardiovasc Drugs. 2011*

despite the utility of the animal models, human models are needed. To better investigate the involvement of the heart tissue in DMD progression, we decided to use hiPSC derived cardiomyocytes to develop a human model of DMD associated dilated cardiomyopathy, investigating the electrophysiological impairment associated with the disease at cardiac level.

Human pluripotent stem cell culture and cardiac differentiation

Protocols for this study were approved by the institutional Review Board of the University of Washington. Written consent was obtained from all study participants. The isolation of the human cells and the subsequent reprogramming into iPSC lines was performed conform to the declaration of Helsinki. Urine derived cells from a healthy male donor and a DMD patient were reprogrammed into hiPSC lines (UC3-4 A1 and UC72039) using a lentiviral vector carrying the fundamental reprogramming factors, Oct3/4, Sox2, Klf4 and c-Myc. By using CRISPR-Cas9 technique on UC3/4 cell line, a DMDKO cell line, called UC1015-6, has been generated. This is the isogenic control obtained with the insertion of one base deletion at the 5'-end of the exon 1 of the dystrophin gene, causing the shift of the reading frame so leading to the production of a truncated protein[230]. The pluripotent lines obtained with the 4 reprogramming factors, were then differentiated to cardiomyocytes. For cardiac differentiation we applied a monolayer directed differentiation protocol by including 1X of hESC qualified Matrigel (Corning) in a defined commercially available cardiomyocytes differentiation kit (Thermo-Fisher). hiPSC were seed in 24-well plates as single cells at the density of about 40.000 cells/well for each line in mTeSR medium supplemented with 5µM of ROCK inhibitor (Y-27632 Gibco). When the monolayer of hiPSC cultures reach appropriate confluence rage, around 70%, cardiac induction was started by adding different culture medium. From day 0 to 2 post-induction, we add the medium A, followed then by the medium B from day 2 to 4 and medium C from day 4 to 6. From days 8 to 10 from the beginning of induction (Medium A added) spontaneously beating monolayer cells should be visible, this need to be feed from day 12 until the end of the culture, with RPMI plus B27 (Life Tecnologies) every day. On day 20 post-differentiation cells were replated on a nanopatterned surface to increase the differentiation process.

[230] *Macadangdang J, Guan X, Smith AS, Lucero R, Czerniecki S, Childers MK, Mack DL, Kim DH. Nanopatterned Human iPSC-based Model of a Dystrophin-Null Cardiomyopathic Phenotype. Cell Mol Bioeng. 2015*

Maturations on a nano-patterned surface

Photo-crosslinked poly ethylene glycol diacrylate (PEG-DA) were fabricated using a capillary force lithography (CFL) procedure, as describe in many previews work[231]. A nanopatterned silicon master was used as template for polyurethane acrylate (PUA) mold, used to pattern a polyutrethane-based polymer via UV-assisted CFL. The PUA master mold used for our purpose was designed with paralleled grooves of 600nm and ridges of 1200nm. PU pre-polymer is drop dispensed onto a glass coverslip and the PUA mold is placed on top. For photo-polymerization the mold was cured via UV radiation (wavelength of 250-400nm) and then peeled off leaving behind an AFS for cell culture. For the maturation process, single cells were replated at low density (10.000 Cells/cm^2) onto fibronectin coated nanopatterned surfaces (Invitrogen, 5µg/ cm^2) and long term cultured.

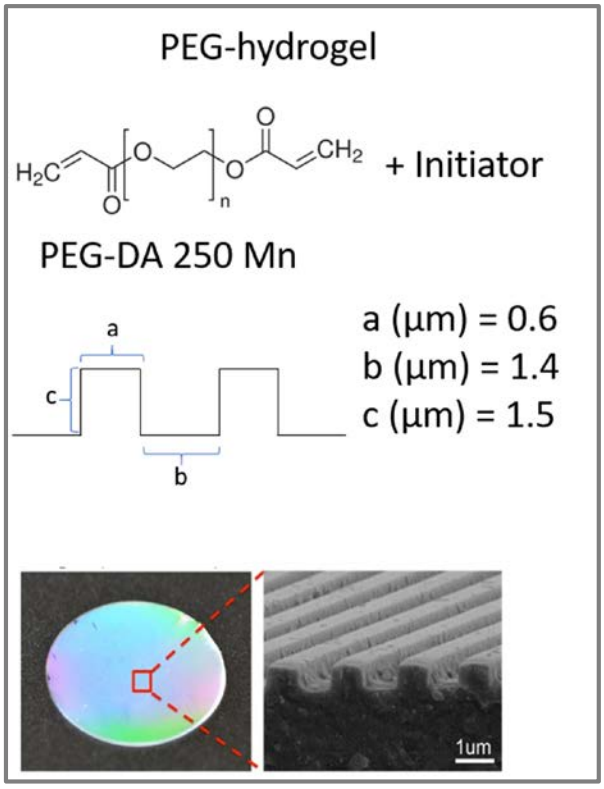


Figure 11. Nanopattern surface used as substrate for cell replating.

The figure shows the structure of the nano-pattern surfaces used in this work as substrate for cell re-plating

[231] *Macadangdang J, Lee HJ, Carson D, Jiao A, Fugate J, Pabon L, Regnier M, Murry C, Kim DH. Capillary force lithography for cardiac tissue engineering. J Vis Exp. 2014*

RESULTS

In this thesis we will discuss different approaches to investigate cardiac arrhythmias associated to inherited cardiac diseases and the efficacy of new and old drugs to treat them. We will start with the cell based approach for drug discovery, to investigate the effects of mexiletine on the *SCN5a-1795insD* mutant sodium channel; then we will focus on the use of human cardiac samples to better understand the mechanisms underpinning the potential effects of disopyramide and to investigate its safeness to treat HCM. Finally, we will evaluate the possibility to use hiPSC to model cardiac diseases and as a platform for drug discovery.

Evaluation of electrophysiological features of sodium current on HEK293 cells transfected with the SCN5a-1795insD or with the wt-SCN5a channel before and after mexiletine treatment

Effects of mexiletine on sodium current-voltage relationship in SCN5a-1795insD or SCN5A-wt

Mexiletine (Mex) usually acts as a sodium channel blocker, but recent studies show how it could potentially act as a chaperone increasing the sodium current density. I decided to compare the effect of mexiletine to vehicle on HEK293 cells transfected with the mutant or the wild type sodium channel, after an overnight treatment or 48h long treatment. The β1-subunit was also transfected together with the α-subunit of the channel as previously described in methods (paragraph: *Heterologous expression and drug treatment*). Our results show that HEK293 cells expressing the SCN5a-1795insD mutant channel and incubated with Mex (500μM) overnight, are characterised by the same peak sodium current density of the vehicle. Moreover, I observed that the I_{Na} density is significantly increased after 48h exposure to Mexiletine (the voltage dependence of activation curves in Fig.12). The SCN5A-wt channel has also been tested to evaluate the effect of 48h treatment with Mexiletine; as reported in Fig.13, Mexiletine mediates an increase of the peak sodium current on wt-SCN5A channel after 48h exposure to Mex but the difference is not statistically significant.

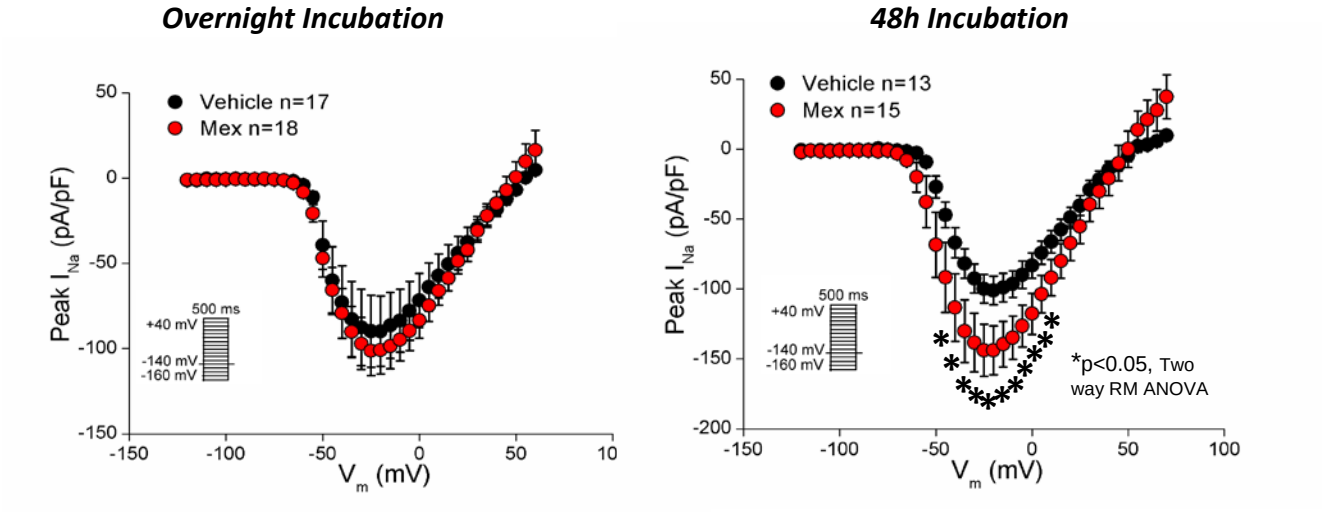


Figure 12. ***Effects of Mexiletine treatment on sodium current-voltage relationship in HEK293 cells transfected with SCN5A-1795insD channel.***

*On the left: effect of overnight exposure to Mexiletine (red dots) or vehicle (black dots) on the IV relationship curve. On the right: the effect of 48h Mexiletine treatment (red dots) versus the effect of 48 vehicle exposure (black dots) on sodium IV relationship. Inserts: voltage protocols; *p<0.05, Two-way RM ANOVA.*

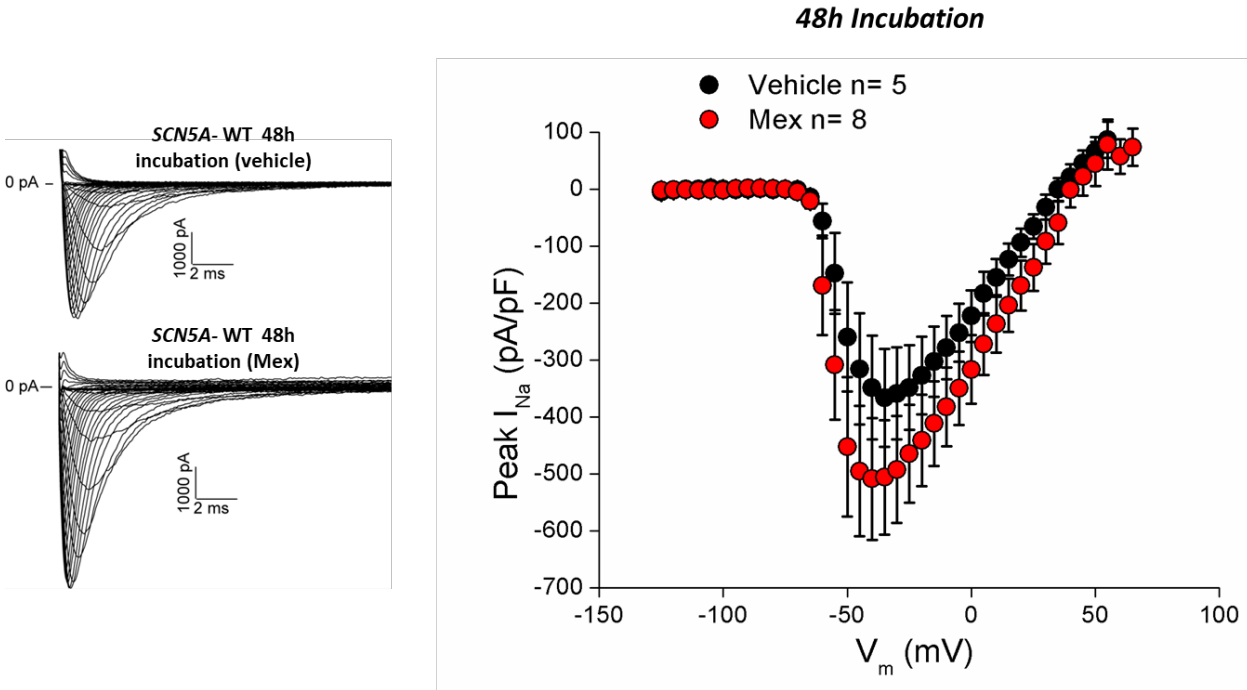


Figure 13. ***Effects of 48h Mexiletine treatment on current-voltage relationship of I_{Na} in HEK293 cells transfected with SCN5A-WT channel***

On the left: representative traces of I_{Na} recorded in wt channel after 48h exposure to Mexiletine (on the bottom) or vehicle (on top). On the right: the average sodium current-voltage relationship after 48h Mexiletine exposure (red dots) or vehicle exposure (black dots)

Effects of Mexiletine on SCN5a-1795insD sodium current kinetics

Mexiletine does not exert an evident effect on the kinetics of the sodium channels, nor of the mutant (Fig 14) or the wild type channel (data not shown) expressed in HEK293 cells. The voltage dependence of activation remains unchanged between the vehicle treated cells (black dots) and the Mexiletine (red dots) treated cells after 48h of incubation or after overnight treatment. Moreover, our data show that the voltage dependence of inactivation and the recovery from inactivation are not affected by the treatment with Mex 500μM. The curves describing the gating properties of the channels are not altered by Mexiletine treatment and there are no significant differences compared to vehicle treated cells.

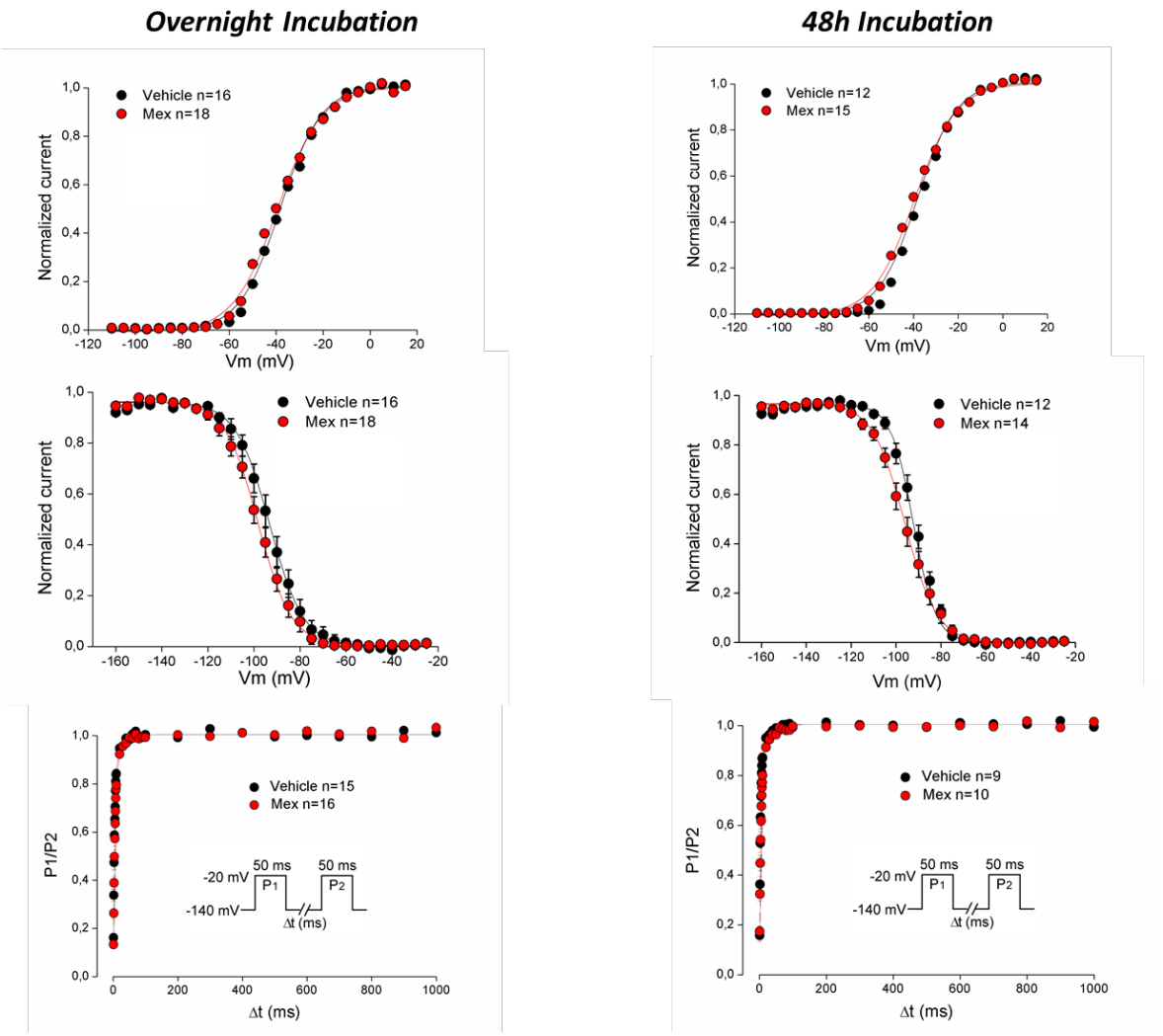


Figure 14. Effects of overnight or 48h Mexiletine treatment on SCN5A-1795insD sodium channel kinetics in HEK293 cells

On the left: representative traces of I_{Na} kinetics, recorded in mutant channel after overnight exposure to Mexiletine or vehicle. On top the voltage dependence of activation, down the voltage dependence of inactivation and in the end the recovery from inactivation. On the right: traces of I_{Na} voltage dependence of activation, inactivation and recovery from inactivation after 48h Mexiletine exposure (red dots) or vehicle exposure (black dots). Inserts: voltage protocols

Disopyramide on human adult cardiomyocytes affected by HCM: effects on AP, cardiac currents and calcium handling

Evaluation of the effects of disopyramide on action potential and ion currents from single human cardiomyocytes.

Human samples of septal myocardium were processed to obtain single cardiomyocytes; then electrophysiological measurements were performed. We collected surgical septal specimen samples from HCM and control patients. We measured AP by whole-cell current-clamp technique and we simultaneously monitored calcium concentration [Ca^{2+}] using a calcium fluorescent probe (Fluoforte Ex/Em 490/515). Sodium and potassium current were measured using whole cell voltage clamp as describe in methods (*Isolation of cells from human cardiac samples*). Each sample is tested with and without Disopyramide.

Disopyramide effects on calcium handling in cardiomyocytes derived from HCM patients

I tested the calcium handling by measuring the intracellular calcium concentration and the calcium transient amplitude at different stimulation frequencies (0.2, 0.5 or 1 Hz) while simultaneously measuring AP. I analysed the kinetics of calcium transient by measuring the 50% of calcium transient decay at each frequency tested. In Fig. 15, we can observe the effects of acute exposure of disopyramide (5µmol/l): it reduces the calcium transient amplitude and the intracellular calcium concentration, accelerating the transient kinetics at 0.2, 0.5 and 1 Hz of stimulation, compared to vehicle. Moreover, I also observed that disopyramide reduced the diastolic Ca^{2+} concentration (Figure 15 A and B).

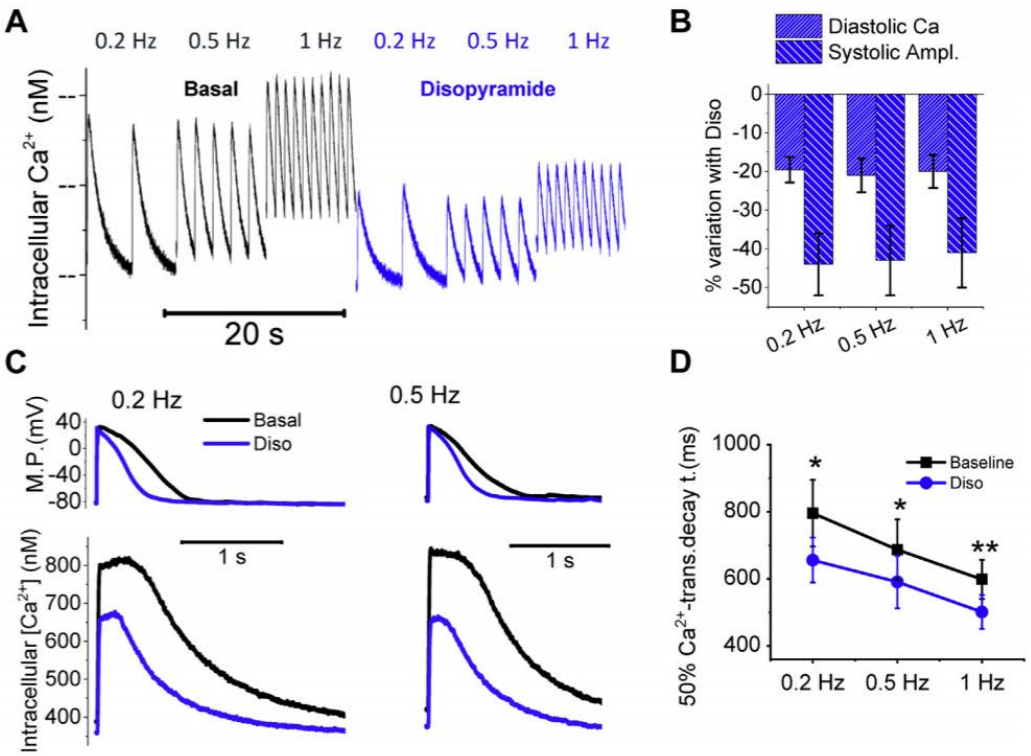


Figure 15: Effects of Disopyramide on intracellular calcium concentration and calcium transient kinetics in isolated cardiomyocytes derived from HCM patients

Calcium transient, recorded from cardiomyocytes derived from HCM patient are reported before (black traces) and after (blue traces) the acute exposure to 5µmol/l *Disopyramide; (**A**) Representative traces of intracellular Ca^{2+} concentration recordings during regular stimulation at 0.2, 0.5, 1 Hz. (**B**) percentage of variation of diastolic and systolic Ca^{2+} concentration from vehicle exposure to disopyramide treatment; (**C**) representative traces of simultaneous recordings of AP and Calcium transients at 0.2Hz on the left and 0.5Hz on the right;(**D**) time from peak to 50% of the decay of Ca^{2+} transient evaluated at all the frequencies tested. Mean, SEM from 26 cardiomyocytes from 7 patients with hypertrophic cardiomyopathy. *0.05 > p > 0.01; **0.01 > p > 0.001; linear-mixed models. MP ¼ membrane potential.*

Evaluation of the effects of Disopyramide on action potential (AP) and arrhythmic events in cardiomyocytes derived from HCM patients

In cardiomyocytes derived from surgical samples of HCM patients, disopyramide is able to shorten the AP duration at all frequencies tested; in particular, the effect is more pronounced at lower rates. The amplitude of AP is significantly reduced after disopyramide treatment; as shown in Fig 16b, the decay phase of AP is hastened at higher stimulation rate and is faster after disopyramide exposure compared to baseline. Disopyramide also reduces the upstroke velocity and decreases the insurgence of early and delay afterdepolarization (EAD, DAD) compared to the basal traces.

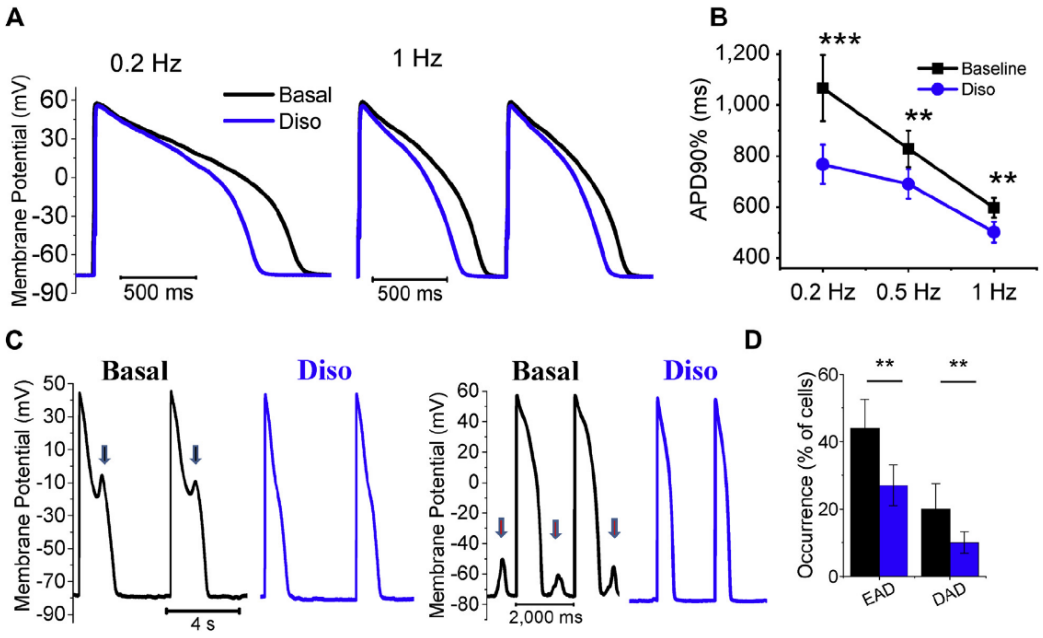


Figure 16: Effect of Disopyramide on action potential in HCM cardiomyocytes

*Representative superimposed action potentials (AP) traces at baseline (black traces) and in the presence of disopyramide 5 mmol/l (Diso) (blue traces), elicited at 0.2 Hz (left) and at 1 Hz (right). (**B**) Action potential duration at 90% of repolarization (APD90%) at baseline (black) and in the presence of disopyramide (blue). (**C**) Representative AP traces at baseline (black traces) and in the presence of disopyramide 5 mmol/l (blue traces), elicited at 0.2 Hz pacing rate. Disopyramide suppresses early afterdepolarizations (EAD) (arrows). (**D**) Percentage of HCM cardiomyocytes showing at least 2 EAD or delayed afterdepolarizations (DAD) during 3 min of continuous stimulation, at baseline (black) and in the presence of disopyramide (blue). (**B, D**) Mean SE from 28 hypertrophic cardiomyopathy cardiomyocytes from 8 patients with hypertrophic cardiomyopathy (ID# 3 to 6, 9, and 11 to 13). **0.01 > p > 0.001; ***p < 0.001; linear-mixed models*

Disopyramide effects on ion currents in HCM cardiomyocytes

In HCM cardiomyocytes ion currents are impaired, leading to a huge remodelling of cell's physiology; I_{Na} and $I_{Ca\text{-}L}$ are significantly increased in affected cardiomyocytes while I_K currents are reduced; in HCM cardiomyocytes disopyramide (5µmol/l) is able to restore a more physiological equilibrium among ions: in particular, it is able to reduce the peak sodium current by 22 ± 4% and the late sodium current by 45 ± 6% (the latest is also the most altered by HCM). Disopyramide is capable to reduce the calcium current ($I_{Ca\text{-}L}$) by 16 ± 4%, and the delayed rectified potassium currents (Fig. 17). The effect of disopyramide on I_K is able to shorten AP in HCM because of the reduction of the aforementioned currents. Conversely, in healthy cardiomyocytes where calcium and sodium currents are not altered, the effect of disopyramide on the potassium currents (reduction) led to AP prolongation. Na^+ currents in HCM cardiomyocytes were measured through patch clamp technique. In the first 10 msec we measured the peak sodium current while the late current was estimated by integrating the residual inward current (50 to 80ms after onset) (21 myocytes tested). The potassium current was recorded at steady state during depolarization at different potentials: the density of steady-state I_K is reduced in HCM cardiomyocytes as compared to control and Disopyramide exert on this current a small inhibitory effect.

Disopyramide Inhibits RyR

Disopyramide also exerts an inhibitory effect on the ryanodine receptors (RYR) like others class I antiarrhythmic. To test the effect of Disopyramide on RYR, we measured the rate of calcium sparks in a transgenic HCM mouse model carrying the R92Q mutation on TnT gene. Cardiomyocytes isolated from the left ventricle of the R92Q mouse were permeabilized and incubated with fluorescent probes. We detected a reduction in calcium sparks rate after disopyramide 5µmol/l exposure than that observed in vehicle-treated cells (Fig. 18).

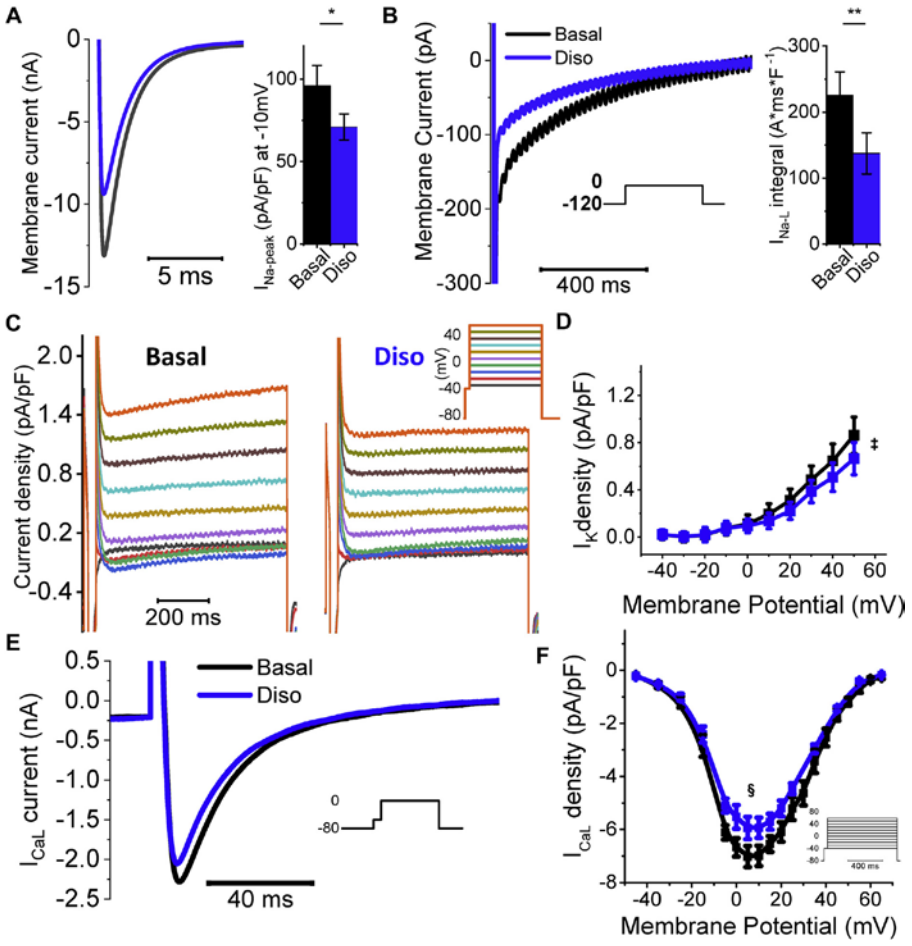


Figure 17: Effects of 5 µmol/l Disopyramide on Ion Channels

*(**A, left**) Representative peak Na current traces elicited at −10 mV from −120 mV resting potential, in a hypertrophic cardiomyopathy cardiomyocyte in the absence (black) and presence of disopyramide (Diso) (blue trace). (Right) Average peak Na+ current (INa-peak) density at −10 mV; effect of disopyramide. (**B, left**) Representative late Na+ current (INaL) traces elicited at -10 mV from −120 mV resting potential, in the absence and presence of disopyramide. (Right) Average integral of the area of the current between 50 and 750 ms after onset of the −10 mV clamp pulse, normalized by cell capacitance, calculated in the absence and presence of disopyramide in hypertrophic cardiomyopathy cardiomyocytes. (**A, B**) Mean ± SEM from 22 cardiomyocytes, 5 patients (ID# 14 to 18). *0.05 > p > 0.01; **0.01 > p > 0.001; linear-mixed models. (**C**) Delayed-rectifier K currents (IK) elicited at different voltages in hypertrophic cardiomyopathy cardiomyocytes (see inset for colour codes), in the absence of disopyramide (left) and in its presence (right). (**D**) Average IK current density in the absence and presence of disopyramide at different voltages. Mean ± SEM from 10 cardiomyocytes, 4 patients (ID# 14, 16, 19, and 20). ‡p < 0.05 for voltages ≥+20 mV, linear-mixed models used to compare each coupled pair of values. (**E**) Representative L-type Ca2+ current (ICa-L) traces elicited at 0 mV from −80 mV resting voltage. (F) Average ICa-L current density in the absence and presence of disopyramide at different voltages. Mean ± SEM from 12 cardiomyocytes, 3 patients (ID# 15, 17, and 18). §p < 0.05 for voltages between −10 mV and +30 mV, linear-mixed models.*

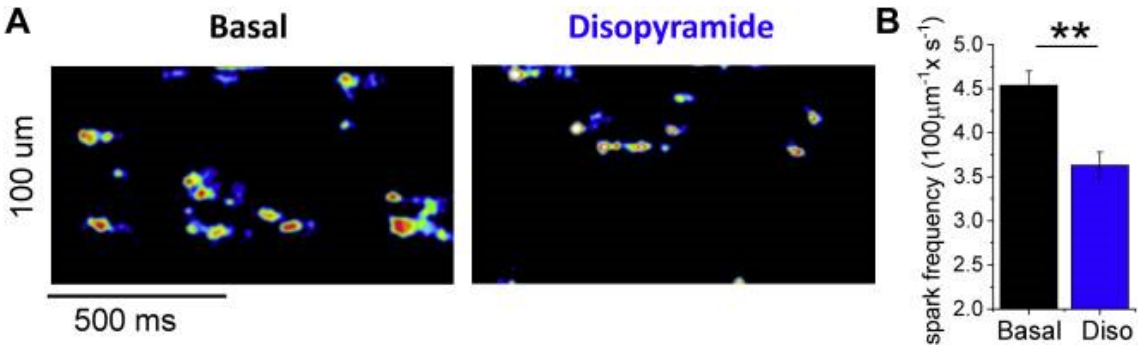


Figure18: Effects of 5 µmol/l of Disopyramide on Ca Sparks in Permeabilized Myocytes

*(**A**) Calcium sparks in permeabilized cardiomyocytes from the left ventricle of transgenic hypertrophic cardiomyopathy mice (R92Q-TnT mutation): the representative kymographic images show confocal line scans traced along the longitudinal cell axis, displaying fluorescence signals from the Ca-selective dye, recorded at a speed of 512 lines/s while bathing the permeabilized myocytes in an intracellular solution containing 150 nmol/l [Ca^{2+}] at room temperature. Spontaneous Ca sparks are visible as spontaneous elevations of local [Ca^{2+}] signal lasting 20 to 40 ms. Representative traces from cells in the absence (left) and presence of disopyramide (Diso) (right) in the bathing solution. Kymographic images are reported in false colour (16-colors lookup table) after thresholding and filtering (Gaussian smoothing with 2px sigma). (**B**) Frequency of spontaneous Ca sparks per 100 µmol/l of scanned line length, in the absence and presence of 5 µmol/l disopyramide. Mean ± SEM from 74 cardiomyocytes (basal) and 63 cardiomyocytes (disopyramide) isolated from 4 mouse hearts. **0.01 > p > 0.001, unpaired Student's t-test.*

Modelling cardiomyopathies associated with systolic dysfunction, using cardiomyocytes derived from human induced pluripotent stem cell: characterization of cardiomyocytes derived from a control line or from DMD line

Characterization of calcium handling during maturation of control cardiomyocytes derived from hiPSC in long term culture

Monolayer of cardiomyocytes derived from control human induced pluripotent stem cells were loaded with the Ca^{2+} indicator Cal-590 for intracellular Ca^{2+} measurements. Cal-590 fluorescence, emitted at 588 nm, was measured during fixed excitation at 573 nm wavelength. Marked cells were submitted to electrical field stimulation at pacing rate of 1 and 2 Hz to simulate heart rate changes. Registration were made at different time point of maturation, 60 and 90 days after differentiation of hiPSC in cardiomyocytes. The calcium transient amplitude increases significantly at day 90 compared to day 60 at both pacing rate tested, 1 and 2 Hz; the latter result highlights the increase capability in stocking calcium of the SR. As we can observe in the lower panels of fig.17, the calcium transient kinetics became faster during maturation. Looking at the rising phase of the calcium transient, represented by the time needed to reach the peak of the transient (Time to peak, TTP), we noticed that velocity in reaching the peak is higher at both 1Hz and 2Hz at day 90 than 60, showing at day 90 a better organization of the sarcoplasmic reticulum. Furthermore, in the control line the TTP is also faster at 2Hz compared to 1Hz at both day 60 and 90, thus explaining the ability of cells to adapt at different pacing frequencies. The decay phase of the calcium transient is represented by the time needed to go from the peak to the 50% of the decay (RT50): the decay phase became faster at day 90 compared to 60 and, as we can observe for TTP, it is also faster at 2Hz than 1Hz, confirming the ability to adapt calcium handling at different frequencies of stimulation and becoming similar to those of human adult cardiomyocytes at day 90.

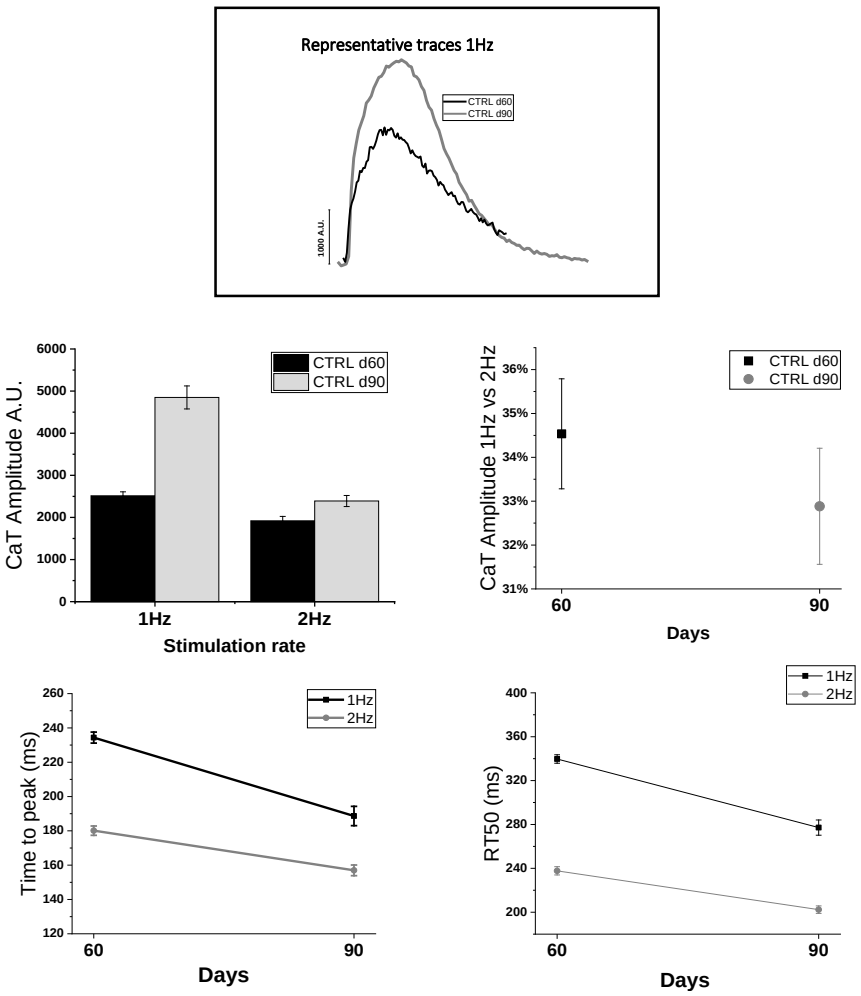


Figure 19. Recording calcium transient in later-stages hiPSC-cardiomyocytes.

Timescale resolution of single hiPSC-cardiomyocytes (hiPSC-CMs) matured on hydrogel-based micropatterned surfaces and measured for calcium transients by fluorescent dyes under electrical pacing (1Hz and 2Hz) at 37°C at [Ca^{2+}] 1.8mM. Simultaneous traces of calcium transients recorded by Cal630 at day 60 (N=3; n=336), and 90 (N=3; n= 165) for calcium transient (CaT) rise (time to peak TTP, ms) and CaT decay (difference of 50% of CaT decay and TTP, RT50, ms) paced at 1 and 2Hz. On top, representative traces of CaT, 1Hz paced at d60 (black) vs d90 (grey) post differentiation.

Data are reported as Means ± SEM; One-way analysis of variance (ANOVA) with a Tukey post-hoc test with statistical significance set at p<0.05 and p<0.01**; NS not significant. N= number of differentiations; n=cells*

Evaluation of sarcoplasmic reticulum development during hiPSC-CM maturation

To evaluate the contribution of sarcoplasmic reticulum to intracellular Ca^{2+} concentration, we applied a post rest potentiation protocol to cells at day 60 and 90. After a short period of stimulation at 2Hz pacing rate, we abruptly interrupted the stimulation for 5s and then we applied again the stimulation. The first peak after the pause reflects the calcium accumulated during the interruption in the sarcoplasmic reticulum, it is, in adult cardiomyocytes, significantly increased in amplitude compared to the last peak before the rest. It describes the contribution of sarcoplasmic reticulum to the cytosolic calcium concentration. As reported in figure 19A, the potentiation of the peak post rest on the pre-pause peak is higher at day 90 than 60. The lack of potentiation at day 60 suggests probably a little contribution of SR to increasing $[Ca^{2+}]_i$ during contraction. In figure 19B the simultaneous registration of calcium transients and AP, shows that both the amplitude of the AP and of the calcium transient, increase after the pause from stimulation. We also applied the caffeine test to evaluate the caffeine induced calcium release from SR: hiPSC cardiomyocytes shows a rapid and large release of Ca^{2+}after rapid exposure to caffeine.

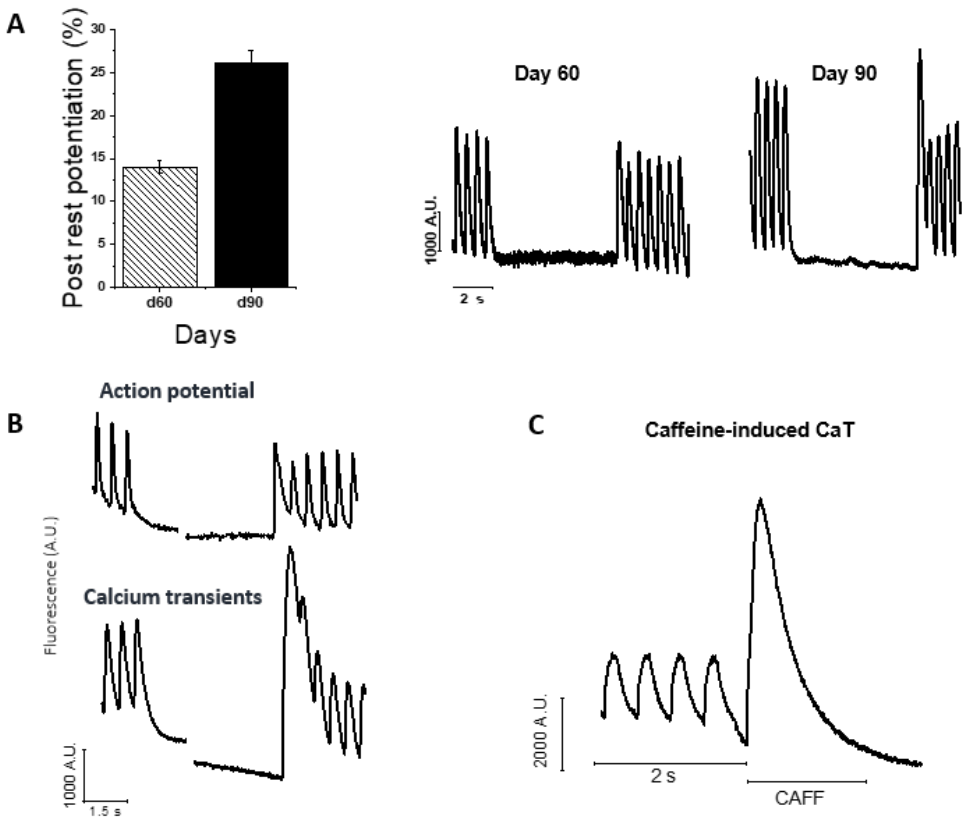


Figure 20. Sarcoplasmic reticulum contribution during hiPSC-CM maturation.

Sarcoplasmic reticulum (SR) contribution in calcium handling maturation was tested by a post rest potentiation protocol and caffeine-induced CaT at multiple time-points. (A) A post-rest potentiation of CaT amplitude was estimated at 2Hz, with a resting pause of 5 s at all time-points (day 60, 75 and 90). The potentiation is calculated from the peak amplitude post-rest and compared with the CaT amplitude before the rest (%). Post rest potentiation is estimated at day 60 and day 90 and presented as day 60 vs. 90. (B) Simultaneous recording of action potential and calcium transient during a post rest protocol (C) Caffeine-induced CaT (10μM) after a series of 2Hz paced CaT (N=2; n=83). Average of caffeine transient amplitude was normalized by the amplitude of steady-state calcium transients at 2Hz prior to caffeine exposure. Interquartile range test; Control d60 N=3; n=336. d90 N=3; n=165 One-way analysis of variance (ANOVA) with a Tukey post-hoc test with statistical significance set at p<0.05 and p<0.01**; NS not significant. N= number of differentiations or patients; n=cells*

Evaluation of action potential kinetics development during hiPSC-CM maturation

On control cardiomyocytes derived from hiPSC, I evaluated the development of action potential at different stimulation rate and time points. I investigated AP at early stages, days 20, 30 and 60 after differentiation, by using the patch clamp technique and AP at d90 post differentiation by using fluorescent probes, (FluoVolt). As the maturation process is going on, AP of hiPSC derived CM resembles the features of the human adult cardiomyocytes, becoming longer and higher in amplitude during cell maturation at each frequency tested (Fig 21B). The resting membrane potential became more negative and the spontaneous activity decreases significantly from day 30 to 60. At d90, the APD50 became longer compared to d60 at 1Hz of stimulation. At 2Hz of stimulation, the APD50 of d90 hiPSC-CMs is shorter compared to 1Hz, showing the ability of cells to adapt APD to different frequencies of stimulation (figure 21D).

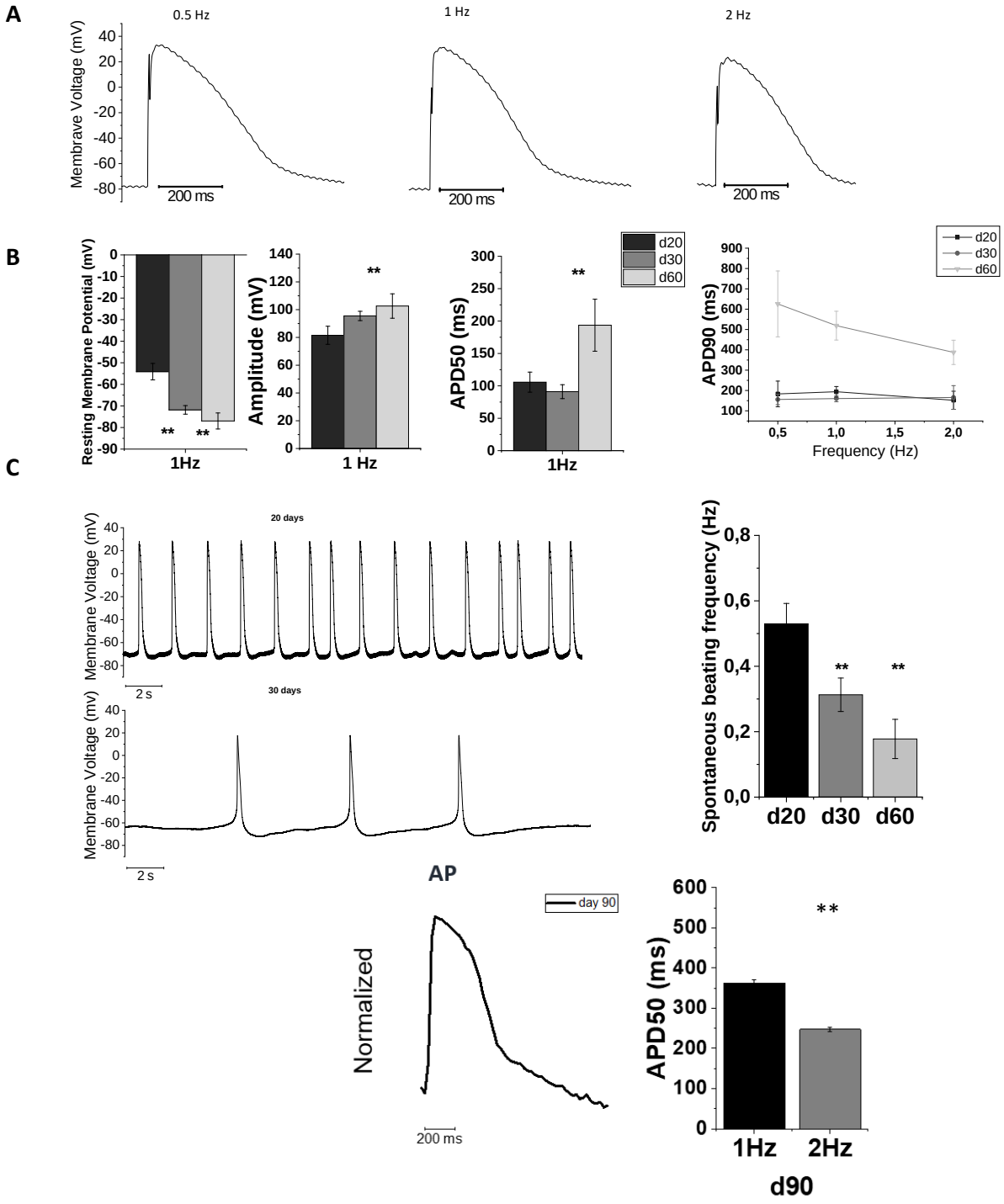


Figure 21. Action potential recording by patch clamp in earlier stages hiPSC-CMs.

Earlier-stages hiPSC-CMs during patch clamp, elicited with short current pulses in current-clamp mode at day 20, 30 and 60. (A) Representative traces at 0.5, 1 and 2Hz of stimulation rate at day 60. (B) Average of resting membrane potential (mV), AP amplitude (mV), time from stimulus to 50% repolarization (APD50, ms) at 1Hz and to 90% of repolarization (APD90, ms) with frequency variation. (C) Spontaneous beating frequency of action potential from day 20 to day 60. (D) A representative trace of AP recorded in hiPSC-CM at d90 post differentiation, at 1Hz of stimulation

using FluoVolt fluorescent probe (on the right) and the mean of the 50% of action potential duration at 1Hz and 2Hz of stimulation. Data are reported as Means ± SEM; One-way analysis of variance (ANOVA) with a Tukey post-hoc test with statistical significance set at p<0.05 and p<0.01**; NS not significant.*

Characterization of Cardiomyocytes derived from a DMD line of hiPSC

As previously explained in methods, we differentiated cardiomyocytes from DMD-hiPSC to study the electrophysiological alterations of human cardiomyocytes during disease progression. Indeed, it is known that DMD also involved cardiac tissue and affected patients usually develop DCM in adult life, that is one of the main causes of death in adult patients. We also differentiated cardiomyocytes from a DMD line obtained by CRISPR-Cas9 genome editing technique applied to a control line of pluripotent cells derived from a healthy donor. We introduced a mutation in the dystrophin gene to prevent dystrophin production and mimic the disease at cellular level.

We worked on cardiomyocytes differentiated from a control line derived from healthy donor, a DMD line derived from a DMD patient and an isogenic DMD line called DMD ΔExon1 obtained through CRISPR-Cas9 to characterize the DMD line as model of disease and to investigate the pathological mechanisms underpinning the insurgence of DCM in DMD patients.

Evaluation of calcium handling in DMD vs CTRL-CMs derived from hiPSC

We first analysed the calcium handling, focusing on calcium transient amplitude and kinetics. We compared the DMD lines to the control line at different time point of maturation (day 60 and 90 after differentiation). The calcium transient amplitude, as already observed for control cardiomyocytes, became larger during maturation. From day 60 to 90, it increases for each line tested, showing the highest contribution of SR to the intracellular calcium concentration at later stages of maturation. Both DMD lines show a significantly smaller amplitude compared to control lines, but their amplitude also became larger during maturation resembling the trend observed for control hiPSC-CM. Looking at the kinetics of calcium transient, our data shows that they are faster in the hiPSC-CM affected by DMD compared to control cardiomyocytes. However, the cardiomyocytes differentiated from the isogenic line (DMD ΔExon1) show slower kinetics at day 90 compare to control and DMD cardiomyocytes. The DMD cardiomyocytes at day 60 post differentiation are faster than control hiPSC-CM in the rising phase of the calcium transient, represented by the TTP. At day 90 the rising phase became faster for CTRL and DMD lines compared to day 60, but the control line remains slowly compared to DMD. Only the cardiomyocytes derived from the isogenic line do not change the velocity in reaching the peak during maturation. Analysing the decay phase of the transient (RT50), we can observe that the control cardiomyocytes remain slower than the DMD at day 60 and 90 but both lines are characterized by faster calcium transient kinetics during cell development. The cardiomyocytes derived from the isogenic DMD lines reverse the trend becoming slower while maturation goes on.

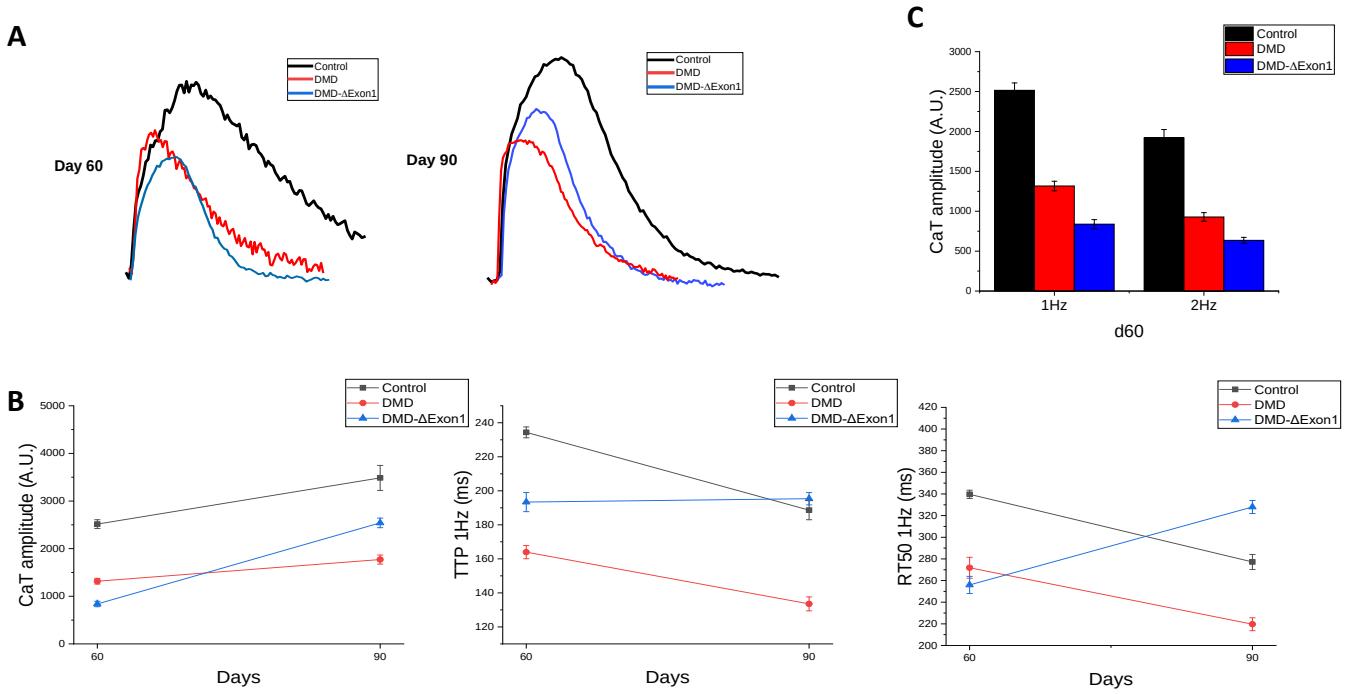


Figure 22. Evaluation of calcium transient differences between control and DMD affected cardiomyocytes derived from hiPSC lines.

Representative traces of calcium transient, measured by fluorescent dye under electrical pacing (1Hz) at 37°C at [Ca^{2+}] 1.8Mm and calcium transient kinetics description. (A) Representative traces of calcium transient recorded at 1Hz, field stimulation, during maturation. Day60 post differentiation traces are reported on the left, day 90 on the right; different colours for different lines, control, DMD and DMD-ΔExon1hiPSC derived cardiomyocytes. (B) Calcium transients recorded by Cal630 at day 60 (N=3; n=336) and 90 (N=3; n= 165); representation of calcium transient amplitude, calcium transient (CaT) rise (time to peak TTP, ms) and CaT decay (difference of 50% of CaT decay and TTP, RT50, ms) paced at 1Hz. (C) Histogram represented calcium transient amplitude at 1 and 2 Hz at day 60 after differentiation in the different cell lines analysed. Data are reported as Means ± SEM; One-way analysis of variance (ANOVA) with a Tukey post-hoc test with statistical significance set at p<0.05 and p<0.01**; NS not significant.*

Sarcoplasmic reticulum contribution to intracellular calcium concentration during DMD derived hiPSC-CM development

A post rest potentiation protocol has been applied to cardiomyocytes differentiated from DMD derived hiPSC and from the isogenic DMD line; a monolayer of cells was stimulated by field stimulation at 2Hz and then a pause from stimulation was applied; after 5 milliseconds the stimulation starts again. During this protocol we recorded the fluorescence emitted by the calcium probe. The first peak after pause reflects the calcium accumulated during the pause in the sarcoplasmic reticulum. Both DMD lines show a slight increase of the post-rest calcium transient amplitude, compared to the last peak before the rest. The post rest potentiation (PRP) of cardiomyocytes derived from the two DMD lines, do not reflect the trend observed in the control line where the increment of the amplitude of post-rest peak became significantly larger during maturation.

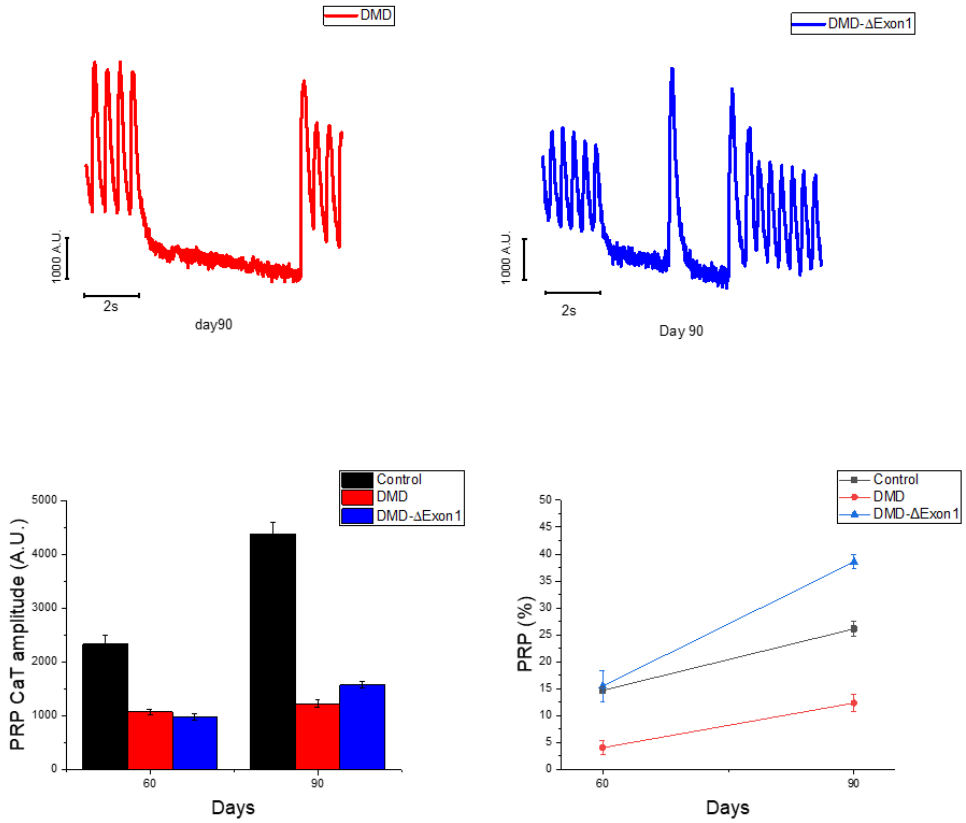


Figure 23. Post rest potentiation of Ca^{2+} transients (Ca-T) from DMD and DMD-ΔExon1 hiPSC-CMs, detected by Cal590 fluorescent dye at 37°C

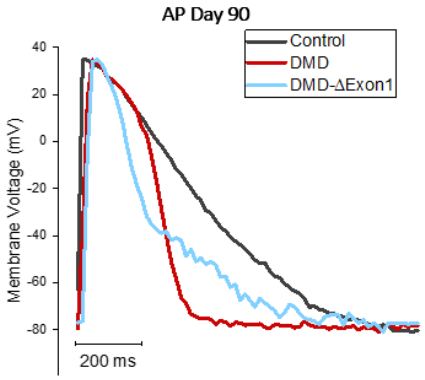
A) Representative traces of Ca^{2+} transients post-rest potentiation from DMD hiPSC-CMs and DMD-ΔExon1- iPSC-CMs, evaluated at 90 days post differentiation B) Evaluation of the

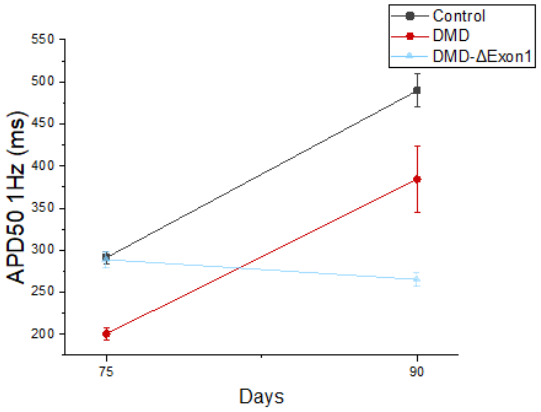
*potentiation of Ca-T after a pacing pause of about 5 seconds (p2 / p1) in control, DMD and DMD-ΔExon1- iPSC-CMs, at different time points of the maturation process (60, 90 days). *p<0.05 and p<0.01 **p<0.01; *<0.05; NS not significant; estimated by one-way ANOVA with Tukey post-hoc test. DMD d60 N=3; n=193. d90 N=4; n=169. DMD-ΔExon1 d60 N=2; n=81. d90 N=2; n=260.*

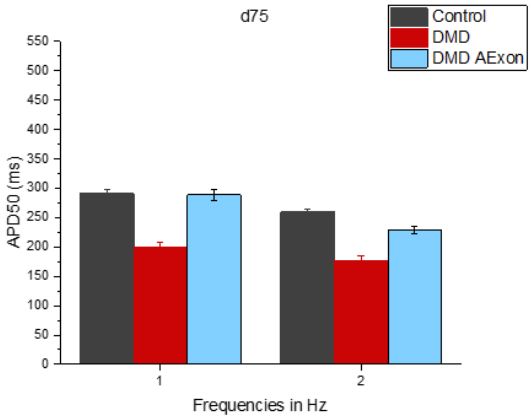
Evaluation of action potential features in DMD vs CTRL cardiomyocytes derived from hiPSC

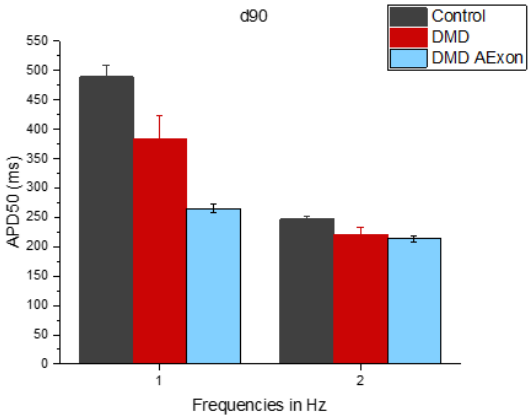
Cardiomyocytes derived from hiPSC were loaded with the membrane voltage sensitive dye Fluovolt (Ex 490nm-Em 516nm) for 30min before measuring the action potential. After loading, cells were perfused with Tyrode solution in the temperature-controlled recording chamber. Cells were stimulated at two pacing frequencies (1Hz and 2Hz) and tested at two different time point of maturation, day 75 and 90 after differentiation. For what concern the kinetics of the action potential, we only evaluated the repolarization phase of AP, analysing the APD50. At 1Hz pacing rates cardiomyocytes at d75 derived from DMD hiPSC line have lower APD50 than control and DMD ΔExon1 hiPSC-CMs; this trend is observed also at 2Hz of stimulation. At day 90 the membrane repolarization of both control and DMD hiPSC-CM became slower, while ΔExon1 hiPSC-CMs do not change the APD50 significantly during maturation. Then, we observed that at day 75 post differentiation, the APD50 does not change significantly between 1Hz and 2Hz of stimulation for all the three lines tested; at day 90, both control and DMD hiPSC-CMs became faster at 2Hz than 1Hz, hastening the AP kinetics thus confirming their ability in adapt cells electrophysiology to different frequencies. Only the ΔExon1 hiPSC-CMs does not show adaptability to different stimulation frequencies during maturation.

We also evaluated the possible correlation between APD50 and calcium transient kinetics (TTP and RT50) during maturation for each cell we measured of control and DMD hiPSC-CMs. We observed a positive correlation between APD50 and CaT kinetics in terms of time to reach the peak of the transient and time to decay of the 50%; in both lines, control and DMD, while the action potential became longer, the TTP and the RT50 of the calcium transient became slower. At the same time, the correlation between the calcium transient amplitude and the duration of the action potential, is negative, so with a prolonged action potential usually we have a smaller amplitude of CaT.

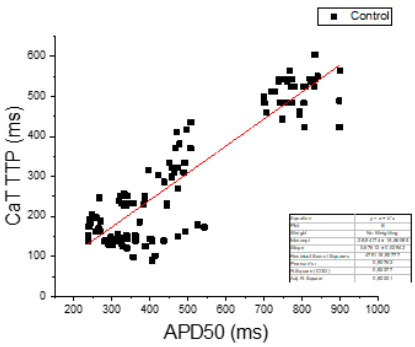

AP Day 90
Control
DMD
DMD-ΔExon1
Membrane Voltage (mV)
200 ms

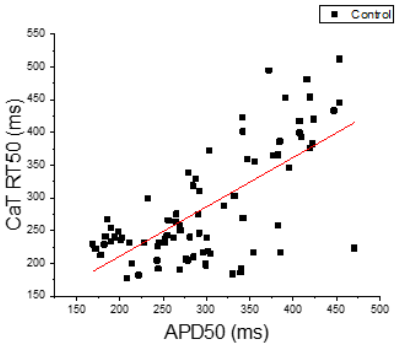

Control
DMD
DMD-ΔExon1
APD50 1Hz (ms)
Days

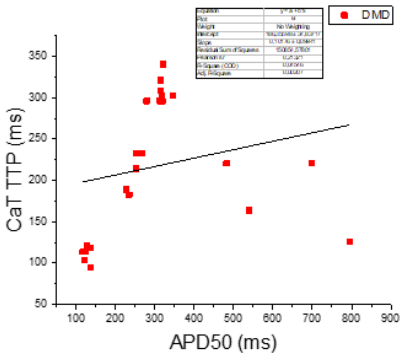

d75
Control
DMD
DMD AExon
APD50 (ms)
Frequencies in Hz


d90
Control
DMD
DMD AExon
APD50 (ms)
Frequencies in Hz

1Hz Day 90


Control
CaT TTP (ms)
APD50 (ms)


Control
CaT RT50 (ms)
APD50 (ms)


DMD
CaT TTP (ms)
APD50 (ms)

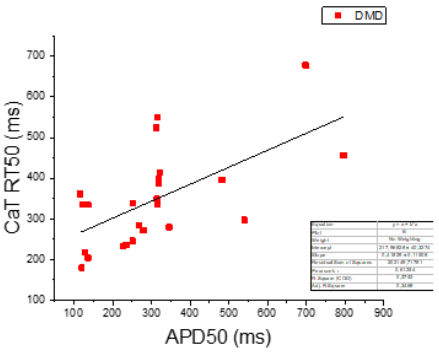

DMD
CaT RT50 (ms)
APD50 (ms)

Figure 24: Action potential kinetics in CTRL, DMD and DMD ΔExon1 hiPSC-CMs and correlations with the calcium transient kinetics

On the top: (Left) representative traces of action potential recorded by measuring the fluorescent emitted by Fluovolt probe; in dark grey the control, in red the DMD and in light blue the DMD ΔExon1 hiPSC-CMs. (Right) 50% of action potential duration at 1Hz is reported for all the line tested at day 75 and 90. In the middle: histograms reported, for all the lines tested, the 50% of action potential duration at 1Hz and 2 Hz at day 75 (Left) and 90 (right) post differentiation. On bottom: correlation between calcium transient kinetics and the 50% of APD for control (black dots) and DMD (red dots) hiPSC-CMs

SUMMARY AND DISCUSSION

Modelling cardiomyopathies has always been challenging for basic science studies; as we discussed above, the term cardiomyopathy refers to a large group of cardiac diseases that involve directly the cardiac contractile tissue, affecting in the end the capability of the heart to work as pump. Despite the features could be various, genetic and non-genetic, all cardiomyopathies have in common the impairment of the excitation-contraction coupling mechanism. This alteration could start from the impairment of the ion channel determining the action potential and the calcium handling, or from a mechanical dysfunction of the structures involved in the contraction; as consequence, the electrical and mechanical function of cardiomyocytes are compromised leading to arrhythmias and cardiac dysfunction. Different cardiomyopathies develop arrhythmias by different pathways: in LQTs or BrS for instance the mutations responsible for the disease usually damage channels function causing alteration in the AP waveform, then reflected in the ST elevation or the QT prolongation, that promote dispersion of repolarization and EAD or DAD, thus triggering lethal arrhythmias. Conversely, HCM causes a large remodelling of the heart tissue, causing the impairment of many cardiac channels (responsible for sodium, potassium and calcium current) but also the alteration of calcium handling and the impairment of the energetic balance of the cardiomyocytes, the loss of T-tubules, the dysfunction of myofilaments regulation and, in the end, the occurrence of fibrotic scar tissue. All these alterations represent a perfect substrate for re-entry circuit and for the occurrence of EAD and DAD, triggering lethal arrhythmias. DCM is another common cardiomyopathy associated with sudden cardiac death; in this case is tricky to understand which mechanism is the main trigger for lethal arrhythmias. Among the potential mechanisms the alteration of sarcomeric proteins function or abnormalities of cytoskeletal proteins (that perturb the actin-myosin interaction and force generation leading to the loss of sarcomeric organization) represent a perfect substrate for arrhythmias. As mentioned above, many different genetic alteration and pathways could be the cause of a cardiomyopathy, but has all types of cardiomyopathies show a common impairment of cardiac ion channels and of the structures responsible for contraction, suggesting that these abnormalities could represent a perfect substrate for cardiac arrhythmias and sudden death.

In this thesis we discussed about different methods to model different types of cardiomyopathies, starting from expression systems, human cardiac samples, down to the novel approach of using cardiomyocytes derived from human induced pluripotent stem cells. Considering the variety of causes and possible developments leading to lethal arrhythmias associated to cardiomyopathies, it is clear that there could not be a single model to design this large group of heart muscle dysfunctions.

Expression systems to design ion channel disease

Expression systems are the first choice to model channelopathies; mutation in single cardiac channels, that alter the gating properties of the channel or its expression on cell surface, are studied using cells easy to be manipulated, in order to obtain the expression of the channel of our interest without external factors that could interfere with the channel function. In these systems operators investigate only the alterations related to the channel of interest expressed on the cell surface, without any other regulatory process.

The *SCN5A* gene encodes the cardiac sodium channel Nav1.5 responsible for the rapid upstroke of the AP; Bezzina and co-workers focused on the evidence that mutations in that gene could be associated to LQT3 or BrS, mediating the increase of the late sodium current and the reduction of the peak sodium current, respectively. Those channelopathies are associated with a high incidence of sudden death. Beside the same gene is involved, the ECG features are very discernible from LQT3 and BrS and never found together, making us thinking that the different pathways responsible for BrS or LQT3 could never coexist. In 1999 Bezzina[232] and co-workers screened a large Dutch family with high incidence of nocturnal sudden death and found that, the prolonged QT interval and the ST segment elevation coexist. Analysing the genetic background of this family, the presence of a mutation in the *SCN5A* gene (insertion of an aspartic acid at the C-terminal) were found in all the affected family members; ECG of all the affected members also showed the typical tract of LQT3 and BrS together, thus leading researchers to correlate the mutation to the presence of a mixed phenotype between BrS and LQT3. The mutation was called 1795insD and has been confirmed as responsible for an overlap syndrome between Brugada and LQT3. The SCN5A 1795insD mutant channel shows a decrease of the peak sodium current, common features of BrS, and an increase of the sustained sodium current, typical for the long QT syndrome. Nowadays, there are poor therapeutic options for patients affected from mixed phenotype associated to SCN5A 1795insD, as BrS is still orphan of a specific pharmacological treatments to prevent arrhythmias and sudden death and LQT3S are treated with non-specific drugs, such as sodium channel blockers of class IB, that are able to reduce the sustained sodium current and consequently shorten the action potential duration. The absence of proper therapies for the two syndromes caused by *SCN5A-1795insD* makes difficult to develop a specific treatment for the overlapped pathology. In

[232] *Bezzina C, Veldkamp MW, van Den Berg MP, Postma AV, Rook MB, Viersma JW, van Langen IM, Tan-Sindhunata G, Bink-Boelkens MT, van Der Hout AH, Mannens MM, Wilde AA. A single Na (+) channel mutation causing both long-QT and Brugada syndromes. Circ Res. 1999*

2010, the group of Silvia Priori[233] discovered that the use of Mexiletine (a sodium channel blocker, antiarrhythmic drug class IB), in use to reduce the prolonged AP in LQT3 patients, could exacerbate the QT prolongation by rescuing the trafficking to the membrane of the mutant sodium channel. This made the administration of Mexiletine rather unsafe for LQT3 syndrome patients, but opened new perspective for the use of Mexiletine in BrS phenotypes. The capability of Mexiletine to block the late sodium current and to increase the peak sodium current by the rescue of the trafficking, make it a potential simultaneous treatment for the gain of function and the loss of function associated to the mutation causing the mixed phenotype between LQT3 and BrS. We decided to evaluate whether the mutation of our interest, 1795insD on SCN5A gene, is susceptible to the chaperone action of mexiletine. We used HEK293 cells transfected with the SCN5A 1795insD channel or with the WT cardiac sodium channel, testing the effect of 500μM mexiletine treatment after overnight or 48h exposure. The overnight treatment does not change the peak current or the kinetics properties in either WT and mutant SCN5A. The 48h treatment on the SCN5A1795insD channel caused an increase of 50% of the peak current and the same trend is present in the WT channel. The increase peak sodium current after 48h incubation, without any changing of the gating properties of the channel, could be associated to an increase number of channels on the cells surface due to an enhanced trafficking of the channel to cell membrane. From our preliminary results, 1795insD mutation on SCN5A gene seems susceptible to Mexiletine, that is able to restore the trafficking of the channel to the membrane, confirming its capability to work not only as a sodium channel blocker but also as a chaperone. We still need to evaluate the potential effects of mexiletine on the late sodium current to understand whether the effect on the peak is also related to the reduction of the sustained sodium current at the same concentration, thus rescuing the mixed phenotype associated to 1795insD mutation on SCN5A. Moreover, we need to assess if the chaperone effect is present at the dosages used in standard clinical therapy.

[233] *Ruan Y, Denegri M, Liu N, Bachetti T, Seregni M, Morotti S, Severi S, Napolitano C, Priori SG. Trafficking defects and gating abnormalities of a novel SCN5A mutation question gene-specific therapy in long QT syndrome type 3. Circ Res. 2010*

Human Cardiac samples to investigate mechanisms and safety of Disopyramide in HCM patients.

Cardiomyocytes derived from human cardiac samples represent one of the best models to gain insights into the disfunctions associated to cardiomyopathies at cellular level and to better appreciate all the alterations related to the whole remodelling process that affects the heart tissue in the presence of a cardiomyopathy. We investigated the effect of disopyramide on fresh isolated living adult cardiomyocytes. Disopyramide is a negative inotropic agent in use since the '80s to treat obstructive HCM; however, the mechanisms of action have not been clarified yet; as a consequence, we established to evaluate its beneficial effects in obstructive HCM patients compared to the effects observed on non-hypertrophic cardiomyocytes.

Disopyramide shows a strong negative inotropic effect on control cardiomyocytes isolated from cardiac samples of non-hypertrophic patients; it inhibits the hERG channels decreasing I_{kr}, thus, together with the slight inhibition of I_{CaL} and I_{NaL}, leading to the prolongation of the APD. In control cardiomyocytes, the QT prolongation due to the prolonged AP duration creates a potential substrate for sustained re-entry arrhythmias, thus suggesting to administer disopyramide with caution. The hypertrophic heart tissue undergoes a large remodelling process during disease progression, that alters many cardiac currents and pathways. A common feature of HCM patients is represented by the increase of late sodium current and L-type calcium current, associated with the reduction of hERG and other potassium currents; these alterations lead to the prolongation of the action potential. The effects of Disopyramide on HCM cardiomyocytes are indeed opposite of those observed in control cells. Disopyramide at 5µM/l shows to be able to shorten APD in HCM cardiomyocytes. The drug exerts a block of the enhanced I_{NaL} and I_{CaL}, without markedly altering I_{Kr}. It altered the balance between repolarizing and depolarizing currents, decreasing the depolarizing currents without a marked decrease of K^+ currents, thus promoting repolarization. As a consequence, Disopyramide determines the shortening of the plateau of the prolonged AP in hypertrophic cardiomyocytes. From our data on in vitro cardiomyocytes, we could expect that the treatment of HCM patients with Disopyramide causes a shortening of the QTc; however, a very slight prolongation of QTc interval has been observed in our HCM cohort of patients treated with Disopyramide. The explanation for this slight prolongation of QT tract (in average a 5.8% rise after 3 months[234]) could be explained considering the spatial dispersion of QTc interval through the hypertrophic

[234] *Coppini R, Ferrantini C, Pioner JM, Santini L, Wang ZJ, Palandri C, Scardigli M, Vitale G, Sacconi L, Stefàno P, Flink L, Riedy K, Pavone FS, Cerbai E, Poggesi C, Mugelli A, Bueno-Orovio A, Olivotto I, Sherrid MV. Electrophysiological and Contractile Effects of Disopyramide in Patients with Obstructive Hypertrophic Cardiomyopathy: A Translational Study. JACC Basic Transl Sci. 2019*

cardiac tissue. The in-vitro analysis was performed only on the hypertrophic cardiomyocytes, isolated from the subendocardial region of the basal septum (the most hypertrophied region), but the heart tissue of a hypertrophic patient also shows non hypertrophic regions where the remodelling could have occurred with a much minor severity, and where cardiomyocytes could be considered similar to healthy cells. The left ventricle wall is heterogeneous, and the spatial dispersion of QTc is highly increased in the hypertrophied heart. The different effect exerted by disopyramide on healthy or hypertrophied/remodelled cardiomyocytes in an heterogenous tissue where both types cells are present, may mediate a slight prolongation of the global QT interval but may also induce a reduction of QT dispersion in the whole heart.

The capability of Disopyramide in shortening the APD in hypertrophic tissue could be associated with a reduction of the risk of arrhythmic events. We observed a reduction in EAD and DAD susceptibility in cardiomyocytes tested and treated with 5µM/l Disopyramide, (Fig 16). While EAD reduction could be directly associated with the APD shortening, DAD reduction does not strictly depend on APD[235]. DADs are triggered by the reactivation of NCX due to the spontaneous release of calcium from the sarcoplasmic reticulum[236]. All the conditions that facilitate the reopening of RYR channels on sarcoplasmic reticulum promote the DAD insurgence[237] . Many of them, like the calcium overload of the SR, the increased diastolic calcium concentration and altered properties of RYR by the phosphorylation of the channels by CaMKII are evident in the hypertrophic cardiomyocytes. Our data showed that Disopyramide is able to reduce the diastolic calcium concentration and the calcium transient amplitude (Fig. 15), thus reducing the spontaneous release of calcium from RYR. Disopyramide directly promotes a stabilization of the RYR channels in the closed state, reducing the occurrence of calcium waves. Together, all these mechanisms could explain how disopyramide could reduce the incidence of spontaneous events in HCM cardiomyocytes, thus exerting a strong antiarrhythmic effect in obstructive HCM.

[235] *Fozzard HA. Afterdepolarizations and triggered activity. Basic Res Cardiol. 1992*
[236] *Landstrom AP, Dobrev D, Wehrens XHT. Calcium Signaling and Cardiac Arrhythmias. Circ Res. 2017*
[237] *Bers DM. Calcium cycling and signaling in cardiac myocytes. Annu Rev Physiol. 2008*

hiPSC-CM to model DMD associated dilated cardiomyopathy.

Human induce pluripotent stem cells (hiPSCs) are a novel tool to design pathologies. hiPSCs are human somatic cells reprogrammed to a pluripotent state thanks to re-expression of transcriptional factors, called Yamanaka factors[238] (Oct3/4 sox2 Myc and Klf4), taking somatic cells back to an embryonic state, becoming capable to give origin to any differentiated cell of an adult organism. These cells are obtained from somatic cells of patients or control subjects, preserving the complete human genome and allowing researchers to develop in vitro realistic representations of the complex patient phenotype, theoretically of every tissue of interest. In fact, hiPSC can generate any cell type though the right differentiation protocol. Since scientists have had the chance to obtain immortalized pluripotent stem cells from somatic cells, they started to use it as model of diseases, for drug screening or even as a therapeutic option in the field of the regenerative medicine for specific pathologies[239]. hiPSC opened many possibilities to overcome the limits of expression systems, animal models or freshly isolated human cells to model pathologies and test therapies. Despite hiPSC presents many advantages, they also show many limitations. Indeed, once the differentiation in the somatic cell of interest is induced through a specific protocol, these cells are characterized by a morphological and electrophysiological immaturity. Scientists are working to improve maturation of cells derived from hiPSCs, developing many differentiation protocols. In this work, we used long term culture associated to a patterned surface to better mimic the extracellular environment, thus promoting cell maturation. We used hiPSC to obtain cardiomyocytes in vitro (hiPSC-CMs): hiPSC-CMs role in cardiac research is crucial, as they allow scientists to have an in vitro model of living human cardiomyocytes, overcoming the difficulties in maintaining isolated cardiomyocytes in culture. Moreover, there is a lot of variability among cells of the same cultures and among cells obtained by different protocols of maturation; for this reason, each model needs to be accurately characterized. As specified above, to obtain cardiomyocytes from hiPSC, we used a control line of hiPSC derived from a healthy donor, a line of hiPSC derived from urine cells of a Duchenne muscular dystrophy (DMD) patient and, in the end, a line of DMD-hiPSC obtained through CRISPR-Cas9 genome editing technique applied to the cells of the healthy donor. Our interest for Duchenne muscular dystrophy is related to the evidence that many DMD patients experience dilated cardiomyopathy, that has become one of the major causes of death in

[238] *Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006*

[239] *Rideout WM 3rd, Hochedlinger K, Kyba M, Daley GQ, Jaenisch R. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell. 2002*

adult patients affected by DMD[240]. We discussed above the role of dystrophin as shock absorber in muscular fibres: it is the interface between extracellular matrix and the contractile apparatus inside the muscular cells, mediating many regulatory pathways and responsible for the resilience of fibres to the mechanical stress of contraction. However, little is known about the mechanism mediating the development of dilated cardiomyopathy and systolic disfunction. Our investigation has the purpose to develop a maturation protocol to reduce the number of immature cardiomyocytes derived from hiPSC and to obtain hiPSC-CMs to gain deeper insights into electrophysiological alterations in the development of cardiomyocytes affected from DMD.

We first characterized cardiomyocytes derived from control hiPSC in terms of electrophysiological properties, calcium handling and action potential features during the maturation period. Thanks to the use of fluorescent probes and a fast high-sensitivity/high-resolution camera, we are able to simultaneously record AP and calcium transients at single cell level. The large number of single cells recorded for each acquisition reduces the intrinsic variability and makes our results reliable and repeatable across different plates. We followed the cardiomyocytes maturation from d30 to d60 post differentiation; during this time, AP and the calcium transient kinetics became closer to those recorded in human adult cardiomyocytes. The AP of control cardiomyocytes derived from hiPSC became longer during maturation, and this prolongation is probably due to the increase of calcium currents during the plateau phase. At the same time, the resting membrane potential became more negative and spontaneous events are reduced, probably thanks to the larger contribution of I_{K1} that keeps the resting membrane potential around -80mV in cardiomyocytes starting from day 60 post differentiation. As it has already been clarified in embryonic stem cell (ESC) derived cardiomyocytes[241], during development the funny current decreases while the delayed rectified potassium currents increases, resembling the changes occurred during fetal development. The modifications in AP and resting membrane potential observed in our results led us to hypothesize that hiPSC-CMs follow the same developmental steps observed in ESC-CMs. In the end, we also recorded the AP of hiPSC-CMs using a fluorescent probe at advanced stage of maturation (d90 post differentiation); the results obtained in hiPSC-CMs were comparable to thus obtained in adult-CMs in terms of APD50. Focusing on the calcium transient amplitude and kinetics, we observed many changes during cell development, comparing hiPSC-CM at d60 and d90

[240] *Adorisio R, Calvieri C, Cantarutti N, D'Amico A, Catteruccia M, Bertini E, Baban A, Filippelli S, Perri G, Amodeo A, Drago F. Heart rate reduction strategy using ivabradine in end-stage Duchenne cardiomyopathy. Int J Cardiol. 2019*

[241] *Sartiani L, Bettiol E, Stillitano F, Mugelli A, Cerbai E, Jaconi ME. Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: a molecular and electrophysiological approach. Stem Cells. 2007*

post differentiation. The calcium transient amplitude became larger and kinetics of its rise and decay became faster, almost resembling that of human adult cardiomyocytes. The increase of calcium transient amplitude during maturation could be associated with an increased contribution of the sarcoplasmic reticulum to intracellular calcium concentration. The faster kinetics in the rise and decay of the transient are the results of the increase SERCA/PLB function associated with the development of the SR and the increase of NCX activity on the cell membrane. To confirm the involvement of the sarcoplasmic reticulum to calcium transient kinetics, the caffeine test and the post rest potentiation protocol have been performed too. The potentiation after stimulation pauses increased during development, and the caffeine induced calcium transient became larger during maturation, thus highlighting the increment of sarcoplasmic reticulum contribution to excitation-contraction coupling. The increased sarcoplasmic reticulum development and contribution to intracellular calcium concentration, the prolonged APD due to I_{CaL} increment and the more negative resting membrane potential due to the reduction of funny currents and the increase of I_{K1}, suggested that with long term culture we could obtain cells that almost resemble the human adult cardiomyocytes. These mechanisms also make cardiomyocytes able to physiologically respond to different stimulation frequencies. Indeed, the AP and CaT became faster at 2Hz (as compared with 1Hz) at d60 and d90 post differentiation, thus suggesting the capability of the cells to response to different stimuli like adult cardiomyocytes. The long-term culture (until day90 post differentiation) associated to patterned surfaces has proved to be effective in ameliorating the maturation of hiPSC-CMs, producing an in-vitro model of cardiomyocytes with electrophysiological features that almost resemble those of human adult cardiomyocytes. This method gave us the chance to study the alterations of cardiomyocytes development in cardiac diseases. We decided to use it to study the dilated cardiomyopathy associated with Duchenne muscular dystrophy. First, we characterized the two DMD line we have used, one derived from DMD patient, the second -DMD ΔExon1- obtained from a healthy hiPSC line through deletion of a DMD gene exon using the CRISPR/CAS9 technique. During the maturation period (from d30 until d90 post differentiation), DMD-hiPSC-CMs followed the same steps of control cardiomyocytes, indeed the AP duration became longer during cells development at 1Hz and 2Hz of stimulation. Moreover, at later stages of the maturation period (d90), APD50 shows better adaptation to different stimulation frequencies. Conversely, APD50 recorded in DMD ΔExon1 shows less adaptability to stimuli. In the DMD line, calcium transient also showed the same trend observed in the control cardiomyocytes, as they became faster in the kinetics of rise and decay and bigger in terms of amplitude of the transient. Even for calcium transient, DMD ΔExon1 does not follow the trend of control and DMD hiPSC-CMs during maturation; indeed, except for calcium transient amplitude that became larger

during development, the time to peak did not change during maturation and the decay phase of the transient became slower at d90. As we did for control lines, we evaluated the SR contribution to intracellular calcium concentration with the post rest potentiation protocol. The potentiation of the calcium transient amplitude in the PR protocol is higher at day 90 than 60 for DMD and DMD ΔExon1 hiPSC-CMs, confirming that during the maturation period the SR becomes able to store more calcium ions and release them when stimulated. If we compare the features of DMD cardiomyocytes affected to control cells, we can appreciate significant differences probably related to the absence of full-length dystrophin. Calcium transient amplitude is much smaller in the pathological cardiomyocytes and kinetics are faster than in control hiPSC-CMs. In fact, DMD hiPSC-CM calcium transients are much faster compared to the control line, where the amplitude of the transient is bigger. The PRP is also smaller in the DMD hiPSC-CM compared to control. These abnormalities in the calcium transient amplitude and potentiation (observed after a post rest protocol) could be linked to an abnormal development of SR and to its inability in stocking calcium, thus, reducing the contribution of SR to the cytosolic calcium concentration. Nonetheless, there is still an increase of PRP during maturation in the affected lines. We also made a correlation for control and DMD hiPSC-CMs between AP duration and calcium transient amplitude and kinetics. In control hiPSC-CM, the shorter the action potential, the faster the calcium transient rise kinetics; the same trend is observed also in DMD hiPSC-CM.

A recent study showed the modelling of DCM associated to DMD using hiPSC-CMs derived from DMD patients, used to investigate the development of the disease at cellular level. They discovered an altered calcium handling leading to an increased cell death; intracellular calcium concentration was found to be increased in DMD cardiomyocytes compared to control cells, but the L-type calcium current was actually reduced. As a consequence, the authors of the study deduced that the intracellular calcium overload is linked to the membrane instability caused by the loss of dystrophin, leading to extracellular calcium entry in the cytosol. They also deduced that the increase calcium concentration triggers the mitochondria death signals, thus promoting cardiomyocytes death[242]. Our findings about a smaller calcium transient amplitude in DMD-hiPSC-CMs could be explained considering the higher intracellular calcium concentration that limits the calcium entry from the SR and T-tubules, thus addressing the altered calcium handling, not to the SR impairment but to the calcium overload secondary to cardiomyocyte membrane fragility.

[242] *Lin B, Li Y, Han L, et al. Modeling and study of the mechanism of dilated cardiomyopathy using induced pluripotent stem cells derived from individuals with Duchenne muscular dystrophy. Dis Model Mech. 2015*

hiPSC-CMs represent a very important source for disease modelling and drug testing, providing researchers with a truly human cell-based system. These data could help in the understanding of the molecular mechanisms behind development of control cardiomyocytes and in the understanding of the pathological alterations that occur during cell development and during disease progression. Moreover, long term maturation of hiPSC-CM could help to identify new target for drug therapy or unknown targetable molecular mechanisms.

CONCLUSIONS

In this work we sought to employ several techniques to model cardiomyopathies, each one with advantages and disadvantages, but each one contributing to our final goal, that is to gain insight into the mechanisms of cardiomyopathies and to identify appropriate drug treatments. The use of HEK293 cells helps us to test the susceptibility of the mutation of our interest (*1795insD-SCN5A*) to Mexiletine, used as a trafficking enhancer for NaV1.5 channel subunits, in addition to the well know function as sodium channel blocker with marked capability of inhibiting late current. This simple method of disease modelling is extremely useful in the field of channelopathies; indeed, our interest was to analyse only the response of the channel to the drug, without any other interaction or interference by other regulatory pathways. Our interest in studying the expression of the channel on the cell surface and the possible alterations in its gating properties makes a more complex model redundant, especially for preliminary studies, like ours. In a study such as ours, an easy and inexpensive model system is to be preferred. HEK293 cells are easy to manipulate, allowing us to express only the channel of interest and, if needed, some regulatory proteins. Most of the components of an adult cardiomyocyte are not expressed on the cell surface, thus leading to a very poor translational value of this model, which does not represent the physiological environment. Anyway, this method could be of great advantage when other models are missing or the subject of our interest is a single protein. Human cardiac samples are a much more complex model of cardiomyopathy, as they express all cardiac channels, regulatory proteins and protein components, and well represent the physiological or pathological condition of the whole heart. On the other hand, human samples are scarcely available and the enzymatic separation methods to obtained single cardiomyocytes could damage some proteins, channels or pathways. Anyway, as they come from subjects affected by specific cardiomyopathies, they make up a complete picture of the condition of the heart muscle in that disease, allowing us to investigate multiple mechanisms and drug interactions, to gain deeper insight into the pathology and to identify new therapeutic targets. We used this model to study the efficacy of disopyramide in reducing arrhythmic events in patients affected by obstructive HCM (OHCM). The heart tissue of affected patients undergoes a large process of remodelling: the expression of cardiac currents changes, leading to alterations in the action potential profile; calcium handling and sodium handling are also affected, the energetic balance is modified and the mechanical function is impaired. All those alterations are hard to be reproduced in an in vitro cultured cell-based model, that is why we employed human cardiac samples to obtain living cardiomyocytes and study the disease. The tissue samples are easily available for this cardiomyopathy, as several OHCM patients undergo surgical septal myectomy to relieve obstruction-related symptoms. Using this model, we could confirm the efficacy and test the safety profile of disopyramide in OHCM patients. Disopyramide is known as antiarrhythmic drug of class IA, responsible of QT tract prolongation, so it is to be

managed with care. We tested disopyramide effects on sodium, calcium and potassium currents and on the action potential and calcium handling profile, in addition to testing its effects on RYR opening probability. Interestingly, we observed that disopyramide has different effects in hypertrophic or non-hypertrophic cardiomyocytes, suggesting that the effects on the drug are influenced by the remodelling of ion channels in diseased tissue. Thanks to the specific changes of ion channel expression and function in the OHCM tissue, disopyramide appears to be a totally safe drug and suggests some possible antiarrhythmic effects, useful for preventing sudden death in these patients. The capability of disopyramide to target different mechanisms responsible for cardiomyocyte excitability and contraction, could only be studied in a complex model like that of human cardiomyocytes where all these targets are present; even rodent transgenic models could not be useful for our purposes because of species differences in ion channel expression and function.

As a final approach, we also obtained and studied cardiomyopathies using the innovative tool of human induced pluripotent stem cell (hiPSC). We derived hiPSCs from a healthy donor (CTRL) or from a patient affected by Duchenne muscular dystrophy (DMD)-related dilated cardiomyopathy, and we then differentiated them into cardiomyocytes obtaining CTRL-hiPSC-CM and DMD-hiPSC-CM. These lines were deeply characterized, in order to investigate the dilated cardiomyopathy associated with the loss of full-length dystrophin. This innovative tool needs to be adjusted, especially in terms of maturations of the cardiomyocytes obtained after the induction of the differentiation. It is still challenging to obtain adult cardiomyocytes and many groups are working to ameliorate the protocols for maturation using different approaches. We decided to use a long-term culture (until 90 days post-differentiation) associated with patterned surfaces that mimic the extracellular matrix, in order to reach a more mature phenotype. Then, we investigated the electrophysiological profile of cardiomyocytes during maturation in terms of calcium handling and action potential profile. This method allowed us to obtain cells that closely resemble the electrophysiological features of adult cardiomyocytes, giving us the possibility of studying the modifications that occurred in the cardiomyocytes over time during their development. Using hiPSCs derived from a DMD patient, we were able to study the progression of the disease during the maturation of cardiomyocytes. Instead of having a snapshot of the disease at a precise point of its progression, using differentiating hiPSC-CMs we can have an idea of what happens during maturation from embryonic to adult cardiomyocytes in control or affected cells. This modern tool gives us the possibility to have a culture of living human cardiomyocytes, that could be used for many purposes; this culture could be easily modified with gene-editing and could represent the future for cardiac disease modelling. hiPSC-CM could overpass the gap of human samples availability and the difficult in culturing adult cardiomyocytes. On the other side, reaching high level of maturation is still challenging and because of the large variability between

different lines is still hard to obtained a reliable and repeatable set of data from different differentiation paths.

These different methods show different advantages, more than everyone the capability of letting us better investigate the efficacy and safety of cardiac drugs, thus leading to ameliorate the current pharmacological treatment or to discover new therapies for diseases that are orphans of a specific treatment. Mexiletine and Disopyramide are both old drugs, not frequently used in the clinical practice. The discovery of new targets and new mechanisms of action opens up the possibility to rethink their use in clinical practice. Multitarget drugs had demonstrated to be more effective in preventing the arrhythmic risk in patients with cardiomyopathies, probably because of the tissue remodelling that occur during disease progression and that involves many different channels and pathways. The capability of a drug to target many of these impaired molecules helps cardiomyocytes to find a new physiological balance to restore the alterations caused by the disease. In this context, the chance of working with accurate models, where all the molecules are represented and all the pathways are running, is crucial. Also, choosing the model on the base of the purpose we want to achieve is critical. For disopyramide, the use of human adult cardiomyocytes affected by OHCM was essential. In the same way, to test the susceptibility of *1795insD-SCN5A* to Mexiletine as trafficking enhancer, a cell-based model (easy to obtain, culture and manipulate) was sufficient and more affordable. In this thesis, we claim that disopyramide could be safely used to treat OHCM. It exerts an possible antiarrhythmic effect, by reducing the insurgence of spontaneous events. Our preliminary data on Mexiletine shows that our mutation is sensitive to its effect in increasing the expression of the channel to the cell surface after chronic treatment. This effect could increase the cardiac sodium current density, restoring the current reduction associated to the mutation. These results confirm the possibility to use the two drugs for specific treatments. The different behaviour that mexiletine and disopyramide could have in the present of different mutations highlight the importance to model the drug treatment on the specific patients we want to treat. The chance to obtain human adult cardiomyocytes from hiPSC, derived from a patient, and to use them as a personal, in vitro, model of disease, could be the feature of drug screening. For that reason, we also focused on the improvement of hiPSC-CMs maturation and we tried to use this model to investigate the alteration associated with dilated cardiomyopathy.