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Review

Modelling genetic diseases for drug development: Hypertrophic cardiomyopathy

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ABSTRACT

Hypertrophic cardiomyopathy (HCM) is the commonest genetic cardiac disease, with a prevalence of 1/500. It is caused by over 1400 different mutations, mainly involving the genes coding for sarcomere proteins. The main pathological features of HCM are left ventricular hypertrophy, diastolic dysfunction and the increased ventricular arrhythmogenesis. Predicting the risk of heart failure and lethal arrhythmias is the most challenging clinical task for HCM patient management. Moreover, there are no disease-modifying therapies that can prevent disease progression or sudden arrhythmic death in HCM patients. In this review, we will illustrate the most advanced research models and methods that have been employed for HCM studies, including preclinical tests of novel or existing drugs, along with visionary future development based on gene editing approaches. Acknowledging the advantages and limitations of the different models, and a critical consideration of the different, often conflicting result obtained using different approaches is essential for a deep understanding of HCM pathophysiology and for obtaining meaningful information on novel treatments, in order to improve patient risk stratification and therapeutic management.

1. Hypertrophic cardiomyopathy: general features

Hypertrophic cardiomyopathy (HCM) is the commonest inherited monogenic cardiac disorder, having a prevalence of one out of 500 in different population cohorts [1,2]. HCM represents a significant cause of sudden death due to ventricular arrhythmias, and is associated with heart failure and atrial fibrillation [1,2]. It has a worldwide distribution, as cases were observed in over 50 countries on all continents [2], and it indistinctly affects males and females [3], as well as subjects of various ethnic origins, with similar clinical course and phenotypic expression [2–4].

Despite a wide variability in the individual pathological features, the hallmark of HCM is represented by left ventricular hypertrophy [1], often asymmetrically distributed, generally involving the anterior basal septum and the anterolateral free wall [5] (Fig.1); in some patients, hypertrophy can be confined to the apex and in a small minority of them, it can be widespread and symmetrically distributed. The appearance of left ventricular hypertrophy can be usually observed at puberty or early adulthood, but the onset of the hypertrophic condition varies, potentially manifesting at birth, during childhood, or developing as late as the 6th decade of life. The burden of left ventricular hypertrophy depends on the thickness of the left ventricular wall: indeed, absolute left-ventricular wall thickness values can vary from mild (13−15 mm) to severe (above 30 mm), with an average of 21–23 mm in different HCM patient cohorts [6,7]. Disease course can be benign, stable, with mild or no symptoms in a significant percentage of patients. Approximately 65 % of HCM patients display symptomatic obstruction of the LV outflow tract at some point during their life, half of them experiencing symptoms only during stress or exercise (inducible obstruction) and the other half with symptoms that are present also at rest [8–10] Obstruction is associated with significant repercussions on prognosis, with significantly increased mortality if untreated [11].

In about 15 % of HCM patients, LV mechanical function starts deteriorating toward systolic dysfunction (hypokinetic progression) or to a severe worsening of LV diastolic function (restrictive progression) [10]. In about one fourth of patients with adverse progression (4% of total patients), end-stage disease develops, leading to terminal heart failure, often requiring transplantation [9,12]. However, the most

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unpredictable and devastating consequence of HCM is represented by arrhythmic sudden death (SD), most commonly induced by sustained ventricular tachycardia (VT), subsequently followed by ventricular fibrillation (VF). Indeed, HCM is a leading cause of arrhythmic sudden death in young population (under the age of 45), with a marked preference for youngster and young adults (age <30 years) [13–15]. The risk of SD is higher in patients where the onset of left ventricular hypertrophy occurs during childhood (pediatric HCM) [16]. SD may occur at any age, and the risk of lethal arrhythmias continues to be present through the whole life, although the average annual risk of SD is relatively low (0.3−0.5% per year) [17]. Moreover, SD in HCM patients is considerably less usual in subjects over 60 years of age, suggesting that in HCM the likelihood of lethal VT is reduced by ageing [15].

1.1. Genetics

Over 1400 autosomal mutations, transmitted in a dominant pattern, have been identified in 11 genes encoding proteins of the thick and thin filament of the sarcomere, or the components of the adjacent Z-discs [18–23]. Most of HCM-causing mutations are unique to individual families, suggesting that this pathological condition is characterized by a high genotype variability. Among the patients characterized by HCM pathogenic mutations, about 70 % have mutations either in the β-myosin heavy chain gene (*MYH7*) or in the myosin binding protein C gene (*MYBPC3*). Mutations in Troponin T (*TNNT2*), cardiac Troponin I (*TNNI3*) and α tropomyosin (*TPMI*) genes assess for about 5 % of cases each [18–23]. Considering the major improvement of the genetic testing in HCM observed in the recent years, Burns and coworkers aimed to study the prevalence of multiple variant genotypes in HCM, evaluating their clinical impact through targeted gene panel tools. To this aim, 758 probands diagnosed for HCM were screened for specific variants through sized gene panels, revealing that multiple variants identified in HCM genes are related to earlier disease onset and worsen survival of HCM patients from major cardiovascular events. In particular, the authors of this study suggest to focus HCM genetic testing on the 8 major HCM genes (*ACTC1, MYBPC3, MYH7, MYL2, MYL3, TNNI3, TNNT2, TPM1*), also extending the screening to additional phenocopy genes as PRKAG2, LAMP2, GLA, and PLN [24]. Despite the current advancements in cardiogenetics and the introduction of modern next-generation sequencing techniques, about 40 % of all HCM cases remain unexplained [25,26], suggesting oligogenic or polygenic mechanisms. Genotype-to-phenotype correlations in HCM are made difficult by the incomplete penetrance of several mutations and by the intrinsic variability of disease presentation, severity and progression, even among different components of the same family presenting the same pathologic variant.

Early studies on large families with a severe disease presentation and high penetrance led to the identification of "high-risk" mutations, such as *MYH7*-R403Q and R453C [27] and *TNNT2*-R92Q/W [28]. Studies on larger unrelated populations partially confirmed the malignant nature of five early-identified "high-risk" mutations (*MYH7*-R403Q, *MYH7*-R453C, *MYH7*-G716R, *MYH7*-R719W and *TNNT2*-R92W) [19] and showed a large variability of outcome among patients carrying the same mutations. Recent studies demonstrated that pathological variants in the genes coding for thin-filament proteins (*TNNT2, TNNI3, ACTC*) are correlated with an higher risk of sudden death during childhood [16] and an increased probability of adverse disease progression during adulthood [29], as compared with the more common thick-filament gene mutations. Despite these differences in outcome between patients carrying thick and thin filament mutations, recent studies on larger HCM populations confirmed that different mutations, even within the same gene, are associated with different disease severity, and confirmed that certain "high-risk" variants (e.g. mutations of the converter region of *MYH7*) are linked with a higher likelihood of SD [30,31]. Finally, patients carrying two or more different disease-causing variants in sarcomeric genes have a higher likelihood of lethal arrhythmias and adverse disease progression [32,33].

1.2. Non-genetic factors

Genetic and non-genetic factors concur to drive the evolution of the pathogenesis of HCM. The phenotypic variability among patients is the end-result of many factors that include oxidative stress, inflammation, apoptosis and dysfunctional regulatory pathways such as autophagy [34,35]. A deeper comprehension of the dysfunctional mechanism involved in HCM pathogenesis is of utmost relevance, especially from a pharmacological perspective. In particular, a crucial role is played by the disproportional energetic demand in HCM [34,35], mitochondrial workload and Reactive oxygen species (ROS) formation. Oxidative stress is a condition characterized by a disproportion between the generation and the neutralization of reactive oxygen species (ROS). The occurrence of oxidative stress in human samples from explanted failing hearts (where the failure is secondary to several cardiomyopathies) [36–38] and its involvement in the pathogenesis of heart failure (HF) was previously reported [39]. Despite scarce information available on the role of oxidative stress in HCM, some evidence in the literature suggests the presence of ROS overproduction in HCM myocardium, causing protein alterations, lipid peroxidation and DNA damage, contributing to cell

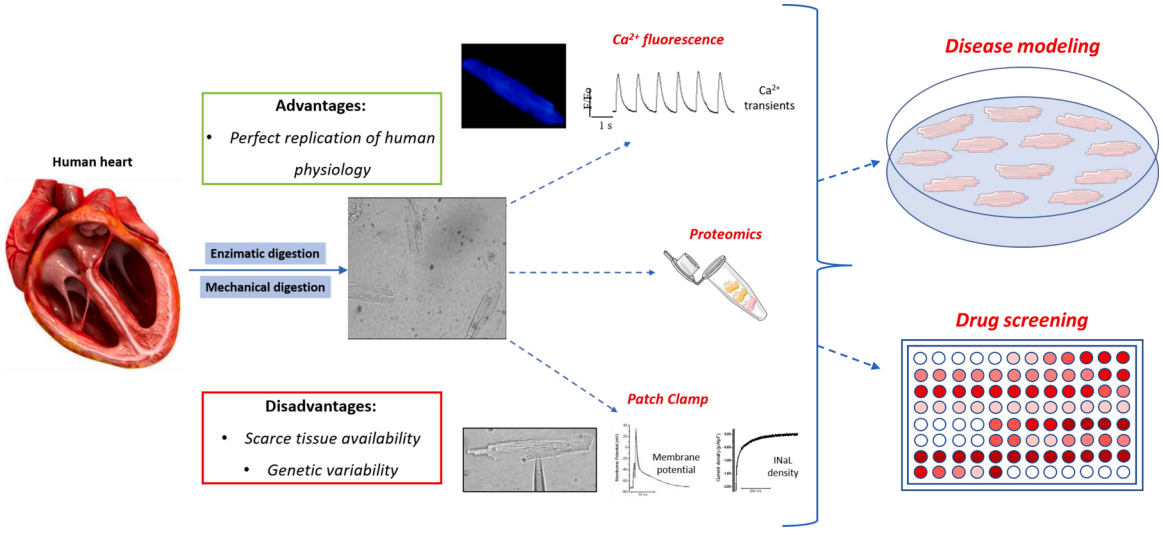

Fig. 1. Human samples for HCM modeling and drug screening.

death [40,41]. A confirmation of the role of oxidative stress in HCM pathogenesis was provided by Dimitrow and coworkers, who observed increased serum levels of 8-iso-prostaglandin F2α in a selected group of patients affected by HCM, compared to healthy controls (35.4 ± 10.2 vs. 29.9 ± 9.9 pg/mL, $p < 0.001$). Moreover, there was a correlation between the obstructive form of the disease and an elevation of oxidative stress by-products, indicative of oxidative stress as an important player in the development of the hypertrophic burden in HCM [40]: indeed, 8-iso-prostaglandin F2α was particularly high in HCM patients with LV outflow obstruction (41.6 ± 12.7 vs. 31.4 ± 5.4 pg/mL in HCM patients without obstruction, $p < 0.0001$). Oxidative stress may be also one of the triggers of low-grade inflammation, feature of the early phase of the development of HCM, and of fibrosis, associated with worse clinical outcomes, through activation of specific signaling pathways [42]. In the heart, ROS also impairs autophagy, i.e., the quality control mechanisms of proteins and nucleic acids, consequently causing senescence and apoptosis [43,44]. The role of autophagy in the maintenance of cardiac homeostasis under stressing pathophysiological conditions has been extensively explored, and several cardiac disorders — including HCM — are indeed characterized by defective autophagy [45,46].

In the last 15 years, the comprehension of HCM patho-mechanisms is consistently improved. This advance in HCM research was made possible by the use of different tools for cardiovascular pathologies modeling, such as human samples and different type of animal models. However, the development of iPSC technique represents the turning point for modeling and studying inherited cardiac pathologies, including HCM. In fact, compared to other research approaches, iPSC method is able to supply a potentially unlimited amount of cells, also preserving the genetic background of the patients, consequently overcoming difficulties and limitations that are intrinsic flaws of other study models.

2. Research models to study the pathophysiology of HCM

2.1. Studies in human samples reveal multiple therapeutic targets

Living cardiomyocytes isolated from fresh human cardiac samples are very informative to gain deeper insights into the specific patho-mechanisms of cardiomyopathies in vitro (Table 1). We recently investigated the electromechanical features of cardiac myocytes isolated from ventricular samples from HCM patients undergoing surgical septal myectomy due severe LV outflow tract obstruction [47,48]. We performed ion fluorescence and patch-clamp experiments to evaluate the electrophysiological abnormalities characterizing the pathogenesis of HCM, by comparing the functional properties of cardiomyocytes isolated from the interventricular septum of HCM patients with cells obtained from samples of non-failing/ non-hypertrophic surgical patients [47,48], observing several abnormalities in the homeostasis of intracellular Na^+ and Ca^{2+} in HCM myocardium [49] (Fig. 1). In particular, patch-clamp experiments showed a significant prolongation of the action potential duration (APD) in HCM cardiomyocytes as compared to controls: APD at 90 % of repolarization was 916 ± 89 ms in HCM cells and 507 ± 61 ms in controls at 0.2 Hz pacing rate, 501 ± 27 ms in HCM vs. 361 ± 42 ms in control myocytes at 1 Hz. The prolonged APD promoted an increased frequency of spontaneous depolarization events (i.e. early and delayed afterdepolarizations – EADs and DADs -), which correlated with the history of non-sustained VT in patients [47]. APD prolongation recorded in pathological cardiomyocytes resulted from a combination of multiple ion current alterations, including the significant reduction of the inward-rectifier K^+ current (Kir2.1/I_{K1}), of the transient outward K^+ current (Kv4.3/I_{to}) and of the delayed rectifier K^+ currents (I_{Ks}, I_{Kr}) recorded in HCM cardiomyocytes, as compared with control cells. Conversely, depolarizing currents such as L-Type Ca^{2+} current (I_{CaL}) and late Na^+ current (I_{NaL}) were both significantly increased in

Table 1
Experimental drug therapies for HCM.

Drug Class	Molecule	Mechanism of action	Experimental models tested on	Clinical trial(s)	OUTCOME of clinical trials
Metabolic modulator	**Perhexiline**	Shift metabolism from free fatty acids to more efficient carbohydrate use	*MYBPC3* mutant mice [109]	Randomized, placebo-controlled, in 46 HCM patients [74]	Improvement of functional capacity and diastolic filling [74]
Ion channel blocker	**Ranolazine**	Late Sodium current (I_{NaL}) inhibition	R92Q-TnT mice [82], hIPSC-CMs [145], human HCM ventricular tissue/cells [47]	RESTYLE-HCM [77], randomized, vs. placebo, in >80 HCM patients	No improvement of functional capacity, lower pro-BNP, less ventr. ectopies [72]
Ion channel blocker	**GS967**	I_{NaL} inhibition	Human HCM ventricular tissue/cells [48]	LIBERTY-HCM (NCT02291237), in 170 HCM pts.	No improvement of functional capacity
Class I anti-arrhythmic drugs	**Disopyramide**	Peak I_{Na} inhibition, I_{NaL} inhibition	Human HCM ventricular tissue/cells [49]	Several studies in pts. with obstructive HCM, including [76]	Reduced LV outflow gradient, less obstructive symptoms and improved survival [76].
L-type Ca_{2+} channel blockers	**Diltiazem**	L-type Ca2+ channel inhibition	R403Q-MHC mouse model [106], hIPSC-CMs [169,190].	Pilot study in preclinical mutation carriers [107]	Delayed the progression of diastolic dysfunction and ventricular thickening [107]
L-type Ca_{2+} channel blockers	**Verapamil, Nifedipine, Amlodipine**	L-type Ca_{2+} channel inhibition	hIPSC-CMs [169,190].	A few old studies with verapamil. Dihydropyridines are contraindicated in obstructive HCM	
Anti-oxydant	**N-Acetyl-Cysteine (NAC)**	Oxygen radical scavenger	*TPM1*-HCM mouse model [108], *MYH7*-HCM rabbit model [128]	Pilot study on 42 patients [130]	Minimal changes of the indices of cardiac hypertrophy or fibrosis [130]
Immuno-modulator	**Rapamycin**	Induction of autophagy	*MYBPC3* mouse (46)	No studies in HCM patients.	No clinical studies
Negative inotropic drugs	**Mavacamten (MYK-461)**	Allosteric inhibition of cardiac Myosin ATP-ase	*MYH7*-HCM mouse model [110], R403Q porcine model [150], feline with HCM [155], hIPSC-CMs (*MYBPC3* mut.) [198], hIPSC-CMs (*MYH7* mut.) [199]	A completed open label phase 2 trial [156], a multicenter phase 3 trial on obstructive patients (EXPLORER-HCM) [157] and a pilot trial on non-obstructive patients [158]	Reduction of LV outflow gradients and obstructive symptoms [156], limited reduction of ejection fraction. EXPLORER-HCM confirmed these positive results. In non-obstructive pts, it reduced plasma pro-BNP and TnI [158].
Statins	**Atorvastatin**	Reduction of LV fibrosis	Rabbit HCM model [129]	A pilot study on 32 HCM patients [131]	No changes of the indices of fibrosis or LV hypertrophy [131]
Anti-arrhythmic drugs	**Mexiletine**	Peak I_{Na} inhibition, I_{NaL} inhibition	hIPSC-CMs (Arg663His-*MYH7* mut) [169].	No studies in HCM patients.	No clinical studies

HCM cardiomyocytes, compared to control cells [47,49]. I_{CaL} density in HCM cardiomyocytes was 7,02091 ± 27 pA/pF, while it was 5,54657 ± 0,42031 pA/pF in control cardiomyocytes ($p < 0.05$); I_{NaL} integral was 195,65 ± 35,21 A*ms*F^{-1} in HCM cardiomyocytes, vs. 74 ± 27 A*ms*F^{-1} in control cells ($p < 0.01$) [47]. We noticed that the myocardial levels of mRNA coding for K^+ channel subunits were all significantly reduced, suggesting that K^+ channels were down-regulated at gene expression level [47]. This modulation exerted at the transcriptional level [50] is likely to be influenced, at least in part, by the enhanced activity of Ca^{2+}/calmodulin-dependent protein-kinase II (CaMKII) in ventricles of HCM patients, via altered histone-deacetylase (HDAC) activation and function [47,51]. Besides the transcriptional effect mediating the down-regulation of K^+ channel expression, the sustained activation of CaMKII plays a widespread role in driving the mechanisms of disease in the HCM myocardium, since it has been observed to promote cardiomyocyte remodeling and dysfunction by direct impairment of intracellular Ca^{2+} homeostasis. Specifically, we and others hypothesized that a sustained increase of $[Ca^{2+}]_i$ is directly caused by sarcomeric mutations (through the increased myofilament Ca^{2+} sensitivity or the impaired energetics and the associated dysfunction of SERCA), causing an initial hyper-activation of CaMKII. Once activated, CaMKII is characterized by a transition to an active auto-phosphorylated state, increasing the activity of CaMKII itself [52] and consequently potentiating the phosphorylation of all the downstream targets (ryanodine receptors, Ca^{2+} channels, Na^+ channels, phospholamban) [53–55]. The increased activity of CaMKII has been identified in animal models and human samples with cardiac hypertrophy and/or diastolic dysfunction [53,54,56]. In fact, we noticed that the auto-phosphorylation of CaMKII was 3.5-fold higher in human HCM samples from patients undergoing surgical myectomy, as compared with controls [47]. L-type Ca^{2+} current is also markedly affected by the activation of CaMKII: in fact, we also observed that in HCM cardiomyocytes the inactivation phase of I_{CaL} was slower, through increased phosphorylation of the L-type Ca^{2+} channel β-subunit, [47,48,57]; this led to increased and prolonged systolic Ca^{2+} entry and contributed to the accumulation of $[Ca^{2+}]_I$ during the diastolic phase. The increased I_{CaL} density recorded in HCM cells [47] is also caused by the slightly increased levels of CaV1.2 protein, which may contribute to the genesis of EADs. Another deeply altered ion current in human HCM cardiomyocytes is late sodium current (I_{NaL}), which was found consistently increased in human HCM cardiomyocytes we obtained from surgical samples. The increased I_{NaL} directly contributes to APD prolongation [47], in line with other human and animal models of hypertrophy [58,59]. In HCM myocardium, CaMKII plays a fundamental role in the increase of I_{NaL} [60,61]. In fact, the increased phosphorylation levels of cardiac Na^+ channel (NaV1.5) mediated by CaMKII [47] impair current inactivation. The role of the aforementioned ion current changes in the prolongation of APD observed in human HCM cardiomyocytes was also validated by mathematical models [47,62,63].

CaMKII activity is also improved by pro-oxidant conditions [64]. Erickson et al. [65] showed that oxidation of paired methionine residues in the regulatory domain leads to stimulation of CaMKII function in the absence of Ca^{2+}/CaM, supporting an independent role of pro-oxidant environment, such as un-scavenged H_2O_2. Among the causal factors, angiotensin II (AngII) may activate CaMKII via NADPH oxidase. Unpublished data obtained from our recent studies confirmed the presence of a high level of oxidation of CAMKII in myectomies from HCM patients.

Besides studying the phenotype of single cardiomyocytes, we also employed human ventricular samples from obstructive HCM patients undergoing myectomy to dissect single intact trabeculae, which were used for recordings of isometric force under controlled mechanical conditions and specific pacing protocols [47–49]. In summary, we identified a slower kinetics of twitch relaxation and a higher diastolic tension at high pacing rates in HCM vs. control trabeculae. At variance with trabeculae from failing hearts, positive inotropic responses to increased pacing frequency and β-adrenergic stimulation were preserved in HCM trabeculae [47,48]. Intact trabeculae, such as single viable cardiomyocytes, need to be obtained from fresh samples and can therefore be studied only for a relatively short time after the sample is obtained from the operating room. However, ventricular tissue from HCM patients can be snap frozen in liquid nitrogen and used at a later time to perform mechanical studied in skinned (demembranated) preparations (i.e. myocardial preparations where all membranes are dissolved using detergents). These include (i) skinned trabeculae/large tissue strips [34,47,66,67] (used to assess myofilament ATPase rate using enzymatic assays), (ii) skinned single cells/bundles [68,69] (used to assess myofilament length-dependent activation and calcium sensitivity of force generation) and (iii) single myofibrils [34,35,67,70] (used to study cross-bridge attachment and detachment kinetics). Studies on human skinned preparation from HCM patients, conducted in the labs of Prof. van der Velden and Prof. Poggesi with the aforementioned techniques, led to important insights on the pathogenesis of myofilament dysfunction in HCM. In particular, they found that most *MYH7* and *MYBPC3* mutations, as well as some *TNNT2* mutations, are associated with a faster cross-bridge cycling rate, that in turn determines a lower maximal force and an increased ATP consumption per unit of force produced (i.e. increased energy cost of contraction). Indeed, HCM is known to be characterized by metabolic impairment caused by an increased energetic cost of myocyte contraction [35], that could significantly contribute to the pathophysiology of the disease [71]. Moreover, the consideration that the levels of high-energy phosphates in the myocardium are significantly affected even in asymptomatic HCM [72] and in the pre-hypertrophic stage of the disease [71] strengthens the concept that the energy deficiency in HCM plays a leading role in the onset and progression of cardiac remodeling. Considering high energy requirements of the heart, cardiac energy deficiency is supposed to represent a contributory cause of the early diastolic relaxation slowing (energy dependent phenomenon correlated with exercise limitation) typical of HCM [73]. The role of energy deficiency as a determinant of HCM-related patho-mechanisms [35] was further investigated by Abozguia and coworkers, who tested perhexiline in an attempt to shift metabolism from free fatty acids to a more efficient use of carbohydrates [74] (Table 1). In particular, the authors of this study evaluated the potential effects of perhexiline on exercise performance in a cohort of 46 patients with symptomatic exercise limitation (peak Vo_2 <75 % of predicted) caused by nonobstructive HCM. Patients enrolled in this study were randomized to perhexiline 100 mg (n = 24) or placebo (n = 22). The rationale of the clinical trial was based on the ability of perhexiline to increase exercise capacity through enhancement of cardiac energetics and improvement of LV relaxation in HCM patients. Myocardial ratio of phosphocreatine to adenosine triphosphate, an established marker of cardiac energetic status (measured by ^{31}P magnetic resonance spectroscopy), left ventricular diastolic filling (heart rate normalized time to peak filling) at rest and during exercise (measured through radionuclide ventriculography) and peak Vo_2 were assessed at baseline and at the end of the study (4.6 ± 1.8 months). Perhexiline confirmed the leading hypothesis according to which altered myocardial energetics drives the major pathophysiological changes occurring in HCM myocardium. Indeed, perhexiline augmented myocardial ratios of phosphocreatine to adenosine triphosphate (from 1.27 ± 0.02 to 1.73 ± 0.02 versus 1.29 ± 0.01 to 1.23 ± 0.01 in placebo-treated patients; $P = 0.003$), normalized time to peak filling (nTTPF) between rest and exercise (0.11±0.008 to 0.01±0.005 s. versus 0.15±0.007 to 0.11±0.008 s. in placebo-treated patients; $P = 0.03$) and improved the functional capacity of HCM patients (tested with cardiopulmonary stress test). Therefore, the present study suggests that perhexiline positively impacts on the quality of life of HCM patients by significantly impairing the metabolism of free fatty acids and serum glucose [74].

Living cardiomyocytes isolated from human cardiac surgical specimens also represent an effective tool to test novel pharmacological

molecules, thus evaluating their potential therapeutic effects (Table 1). Considering the multiple ion current alterations observed in HCM (the increased I_{NaL} is one of the most important contributors to the electrophysiological and $Ca^{2+}{}_i$ alterations occurring in ventricular myocytes) [47,49] and their resulting effects in the pathomechanisms of the disease, an adequate therapeutic approach could be played by the use of molecules able to affect ion channel kinetics. As consequence, we tested whether I_{NaL} blockade with **ranolazine** (10 μmol/L) could represent an effective option to reduce the electromechanical dysfunction in HCM myocardium. At 10 μmol/L, ranolazine reduced I_{NaL} integral from 195, 65 ± 35,21 A*ms*F^{-1} to 77,29 ± 18,21 A*ms*F^{-1} in HCM myocytes ($p < 0.01$) [47]. We observed that ranolazine, through decrease of intracellular Na^+ overload, restored Ca^{2+} extrusion through the forward mode of Na–Ca exchanger (NCX) and reduces Ca^{2+} influx via NCX reverse-mode, thus determining an acceleration of Ca^{2+} transient kinetics and mediating a reduction of diastolic $[Ca]^{2+}{}_i$. In particular, I_{NaL} block by ranolazine significantly reduced APD in HCM cardiomyocytes: APD at 0.2 Hz was 946 ± 95 ms at baseline and 707 ± 71 ms in the presence of ranolazine ($p < 0.01$); at 1 Hz, APD was reduced from 557 ± 44 ms to 443 ± 32 ms ($p < 0.01$). In addition, ranolazine halved the rate of EADs, highlighting the antiarrhythmic potential of ranolazine in this disease. Moreover, via normalization of intracellular Ca handling and diastolic Ca, ranolazine may improve diastolic function in HCM patients [47]. Additionally, DADs were reduced by 60 % in the presence of ranolazine. To confirm that these beneficial effects exerted by ranolazine on HCM cardiomyocytes [47] were directly mediated by I_{NaL} inhibition (and not by potential pleiotropic effects of the drug, such as the stabilization of ryanodine receptors [49]), we tested the selective I_{NaL}-inhibitor, **GS-967**, in isolated human HCM cardiomyocytes [48]. In HCM myocardium GS-967 exerted effects that were qualitatively and quantitatively similar to the effects of ranolazine in the same cells (i.e. I_{NaL} reduction, accelerated AP kinetics, diminished rate of arrhythmogenic EADs, decreased diastolic $[Ca^{2+}]$, suppression of DADs, acceleration of Ca^{2+}-transient kinetics). However, these effects were observed at a 20 times lower concentration (10 μM ranolazine vs. 0.5 μM GS-967), suggesting that GS-967 has an increased selectivity for I_{NaL} and a higher potency of inhibition. We observed that **disopyramide**, a Class I antiarrhythmics drug, was also capable of blocking I_{NaL} in HCM cardiomyocytes [49]. Disopyramide is commonly used in obstructive HCM due to its negative inotropism [75,76]. This effect results from the sum of different molecular actions exerted at cellular level. Indeed, disopyramide is able to slightly reduce $I_{Ca\text{-}L}$, an effect that is, at least in part, responsible for the decrease of systolic intracellular $[Ca^{2+}]$ in the cardiomyocyte. However, we observed that the concurrent inhibition of peak and late I_{Na} by disopyramide is also necessary for its negative inotropic action [49]. Finally, disopyramide interacts with ryanodine receptors, stabilizing them and reducing their probability to open, thus decreasing the amount of systolic Ca^{2+} release. Disopyramide was also observed to exert an anti-arrhythmic effect in HCM cardiomyocytes: I_{NaL} and $I_{Ca\text{-}L}$ inhibition by disopyramide mediates the reduction of AP duration, ultimately shortening AP plateau duration and thus mediating a reduction of arrhythmogenic triggers in HCM cardiomyocytes. Indeed, disopyramide reduced EADs, a direct consequence of AP shortening, and abolished DADs, thanks to the reduced diastolic $[Ca^{2+}]$ and SR Ca^{2+} content, as well as to the stabilization of the closed state of the RyR [49] (Fig. 1).

In parallel with studies on intact cardiomyocytes, we also tested ranolazine, GS-967 and disopyramide in intact and skinned trabeculae, evaluating their effects on mechanical myocardial performance in human HCM myocardium [47–49]. In brief, we observed that ranolazine and GS-967 slightly accelerated twitch relaxation and lowered diastolic tension at higher pacing frequencies, while twitch amplitude was unchanged. Disopyramide had a concentration-dependent negative inotropic effect, that was paralleled by an acceleration of relaxation.

Despite the promising results obtained in human cardiomyocytes, a multicenter, placebo-controlled study with ranolazine in non-obstructive HCM patients with exercise-limiting symptoms revealed that ranolazine had minimal beneficial effects on exercise capacity [77]; nonetheless, ranolazine reduced circulating pro-BNP and markedly decreased arrhythmogenic ventricular ectopies in patients [77]. Eleclazine, the equivalent of GS-967 developed for use in patients, was tested on over 170 HCM patients in the multicenter study LIBERTY-HCM (NCT02291237); again, no significant beneficial effects on exercise capacity were noted in treated patients (https://clinicaltrials.gov/ct2/show/study/NCT02291237).

Skinned human myocardial preparations from HCM patients can be used to assess the effects of drugs that directly target myofilaments. Although no studies have been published so far testing drugs in human HCM demembranated trabeculae, cells or single myofibrils, several studies are ongoing testing the effects of the selective myosin inhibitor mavacamten (see below) in human HCM samples from patients carrying different mutations.

2.2. Animal models as an invaluable research tool to model cardiomyopathies

2.2.1. Rodent models

Despite their high translational value, studies on myocardial samples from patients with HCM show several restrictions, being limited by the scarce availability of surgical material and the wide heterogeneity among patients (the intrinsic genetic background variations, the various clinical expression and environmental influences) [78]. Moreover, surgical human tissue models are representative of a relatively *advanced* disease stage of HCM, therefore is often impossible discerning primary players, induced by a particular gene variant, from the secondary and tertiary mechanisms resulting from myocardial adverse remodeling.

In the past 20 years, animal models of HCM have been developed as an inestimable research platform for studying HCM pathophysiology, disease progression and for testing new therapies. Animal models can represent an effective solution to the limitations of using surgical human samples, with inbred strains eliminating variabilities in the genetic background. Such models were vital to get insight into the causative role of gene variants in HCM and downstream pathogenic signaling pathways. Additionally, they have provided novel knowledge into potential new interventions for the therapeutic management of the disease. Most of these animal models were initially created in *mice*, as rodent models are characterized by several advantages that facilitate their use in scientific research (Fig. 2).

The first animal model developed to study the pathogenesis of HCM was a mouse line carrying a missense mutation at codon 403 in the α-MyHC gene, corresponding to the human β-MyHC R403Q mutation [79]. The R403Q model showed different pathological features such as myocellular disarray, fibrosis, and diastolic dysfunction, faithfully replicating the pathology observed in human tissues [79]. Moreover, the rate of arrhythmogenic events was considerably increased in this mouse model, as compared with wild type (WT) mice [80]. However, left ventricular (LV) hypertrophy (the hallmark of HCM) was not present in this mouse model. A report from Tyska and coworkers revealed that the R403Q substitution is able to enhance both the ATP-hydrolytic and the mechanical performance of the mutant cardiac myosin, increasing actin-activated ATPase activity and the average force generation. Experiments were performed on whole cardiac myosin purified from a mouse model of familial hypertrophic cardiomyopathy (FHC), carrying the R403Q mutation, to eliminate potential uncertainties associated with protein expression systems. The observed increase of myosin ATPase activity suggest that this mutation may affect the mechanical synchronization between the 2 “heads” of a cardiac myosin molecule, impairing the energy transduction process through a “gain of function” mechanism [81]. A rodent model carrying a similar mutation (deletion of amino acids 468–527 in the actin-binding domain of α-MyHC) also reproduced the landmark histologic features of HCM and showed marked hypertrophy of the left- and right-ventricular walls at 4 months

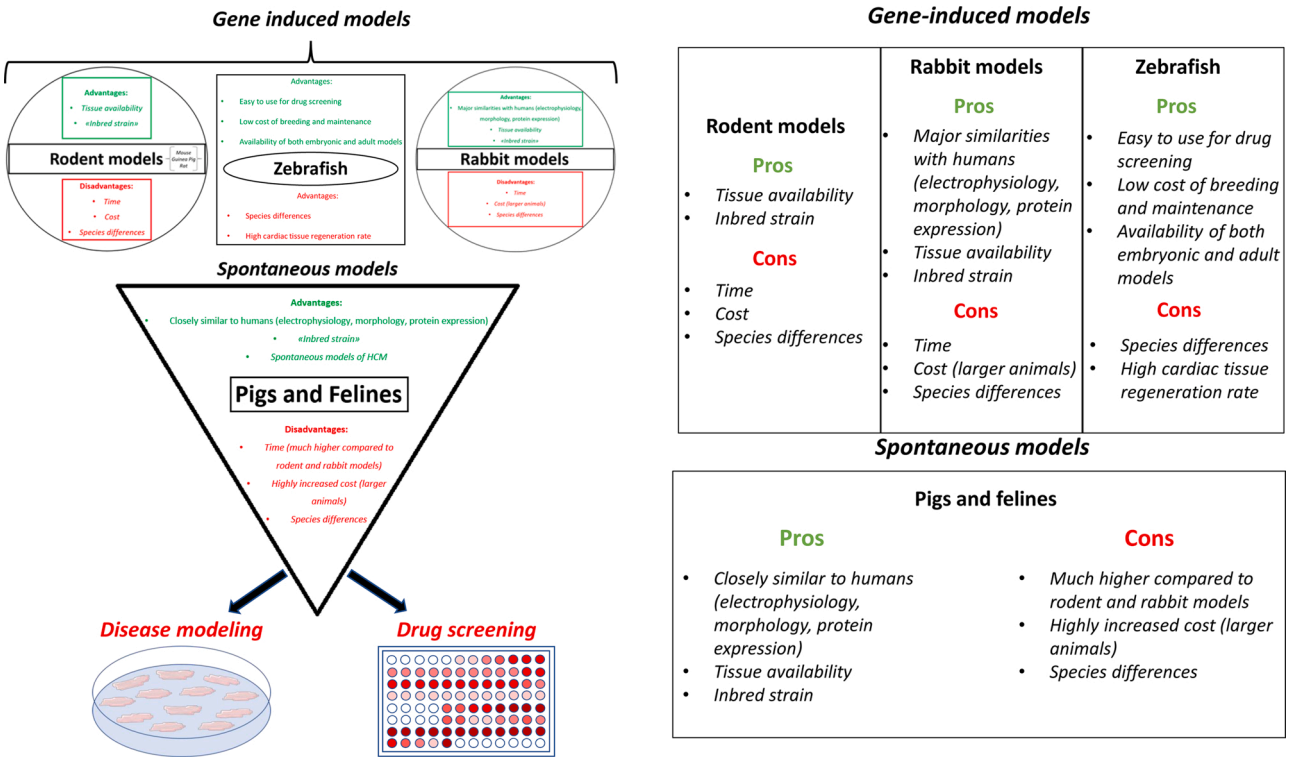

Fig. 2. Transgenic animals to model HCM and for pharmacological testing. This table summarizes all animal models (transgenic and spontaneous models) described in the text to gain deeper insights to pathomechanisms of HCM and to screen novel drugs.

of age, thus representing an efficient model to study the patho-mechanisms of HCM [82]. Another transgenic mouse line expressing a mutated α-MyHC (missing the light chain binding domain) [83] showed the typical histologic features of HCM. Hearts obtained from these mice are characterized by asymmetric hypertrophy, particularly involving the anterior LV wall.

Myosin binding protein-C (MyBP-C) is the most common gene involved in human HCM pathogenesis. Mouse lines carrying a modified MyBP-C with a deletion of the entire myosin and titin-binding domains were developed and studied [84]: these transgenic mice showed light hypertrophy, sarcomere disarray, and altered exercise capacity [85]. Moreover, to study the pathogenic potential of the cMyBP-C-DC10 mutation (the most common mutation associated with the development of HCM), transgenic mice expressing cardiac specific cMyBP-C-DC10mut were generated. Transgenic expression of cMyBP-C-DC10mut is responsible for all the hallmarks of HCM in mice. In this transgenic line the mutated protein lead to an improper assembly of the cardiac sarcomere through a poison-peptide effect. Mice showed a typical HCM phenotype with activation of pro-hypertrophic signaling in the myocardium, determining LV hypertrophy, diastolic dysfunction and contractile abnormalities; moreover, cardiac remodeling was associated with LV fibrosis [86]. Studies conducted in a knock-in mouse model harboring a clinically-relevant point mutation of *MYBPC3* (G > A transition in the last nucleotide of exon 6) led to the concept that diastolic dysfunction and myofilament Ca^{2+} sensitization (two main pathogenic marks of HCM) are initial phenotypic consequences of cMyBP-C mutations, preceding the development of left ventricular hypertrophy (LVH) in HCM. As mentioned above, mutations in Troponin T (*TNNT2*), cardiac Troponin I (*TNNI3*) and α tropomyosin (*TPMI*) account for about 5 % of HCM cases each [87–92]. In the latest years, transgenic rodent models carrying mutations in the α-tropomyosin gene were generated and studied. Among these, a rodent model harboring the D175N α-TM mutation was able to replicate some histo-pathologic features of human HCM, such as myocellular disarray and hypertrophy, with altered contractility and relaxation in vivo. Although the isovolumic phase of systole was normal in these mutant mice, abnormalities were evident with the onset of contraction, consistent with the abnormal fractional shortening observed in echocardiographic studies. While the systolic performance was slightly reduced, the authors described a 40 % reduction of negative dP/dTmax during LV relaxation in mutant mice compared to controls. Moreover, the D175N tropomyosin mutation was observed to cause increased myofilament calcium sensitivity in this mouse model [93]. Different mutations in cardiac troponin T (cTnT) can cause familial HCM. To gain deeper insights into patho-mechanisms of "thin-filament" HCM and to evaluate the main features of the associated phenotypes, Tardiff and coworkers developed transgenic mouse lines expressing 30 %, 67 %, and 92 % of their total cTnT as a missense (R92Q) allele analogous to one found in human HCM. We performed an electrophysiological and morpho-functional analysis of single cardiomyocytes isolated from R92Q hearts to evaluate whether this model could finely reproduce HCM in humans. R92Q hearts showed different common markers of HCM, such as interstitial fibrosis, mitochondrial structural alterations, diastolic dysfunction and hypercontractility [94]. Moreover, cardiac myocytes isolated from R92Q mice were characterized by increased diastolic calcium resulting in an impaired Ca^{2+} handling and slower Ca^{2+} transient kinetics combined with an increased rate of spontaneous arrhythmogenic electrical events [95], replicating data we obtained performing Ca^{2+}-fluorescence experiments on single cardiomyocytes isolated from human HCM samples [41]. Besides various limitations associated to the use of rodent tools to model HCM (summarized at the beginning of 2.2.2 paragraph) and except for AP duration that is shorter in R92Q compared to control cardiomyocytes (in human HCM cardiomyocytes AP kinetics are slower compared to control cells), R92Q cardiomyocytes can accurately reproduce the different pathogenic features proper of human HCM, suggesting that among murine models of HCM, R92Q transgenic mouse could play a leading role to gain deeper insights into cellular patho-mechanisms of HCM.

Sarcomeric mutations in troponin T (TnT) or TnI are associated with a high risk of SCD, even if cardiac hypertrophy is not evident [96,97]. Baudenbacher and coworkers hypothesized that the increased

arrhythmia susceptibility could be independently and directly caused by the raised myofilament Ca^{2+} sensitivity, a direct consequence of the mutations of the sarcomere regulatory proteins. They confirmed this hypothesis by developing transgenic mice carrying HCM-linked TnT mutations conferring strong Ca^{2+} sensitization (TnT-I79N, associated with a high risk of SCD at young age [96,98]), intermediate increase of Ca^{2+} sensitivity (TnTF110I), or minimal changes (TnT-R278C, associated to a better prognosis in patients [99–101]). Myofilament Ca^{2+} sensitization was effectively observed to be a novel and independent mechanism of arrhythmogenesis despite the absence of histological abnormalities (fibrosis, myocardial disarray), commonly considered as the main cause of arrhythmias in HCM. Arrhythmogenesis arose from increased dispersion of ventricular activation at fast heart rates, consequently resulting in reentry arrhythmias [98].

The role of increased myofilament Ca^{2+} sensitivity in HCM pathogenesis was evaluated also in other animal models, such as *guinea pigs*. Murine cardiomyocytes differ from both human and guinea pig for what concerns structure and function. In particular, human and guinea pig are characterized by slow MyHC (β isoform) while murine cardiomyocytes contain predominantly fast MyHC (α isoform) [102]. There are also marked electrophysiological differences: cardiac AP in mice differ consistently in waveform, lacking any appreciable plateau [103]. Robinson and co-workers developed a stable but short-term transgenic cardiomyocyte guinea pig model of HCM through adenoviral expression of HCM-associated variants in the genes encoding for human troponin-T, troponin-I, and alpha-tropomyosin (R92Q, R145G, and D175N respectively), in adult left ventricular cardiomyocytes from the guinea-pig heart. Isolated cells showed different pathogenic alterations that are typical of human HCM, such as abnormal Ca^{2+} transients, increased cytoplasmic [Ca^{2+}] and a clear activation of Ca^{2+}-dependent signaling mediated by CaMKII. Moreover, increased myofilament Ca^{2+} affinity, one of the primary consequences of HCM sarcomeric mutations in humans, was observed to double the total Ca^{2+} buffering by the myofilaments, directly altering intracellular Ca^{2+} homeostasis. This cardiomyocyte model also showed and confirmed a direct correlation between myofilament Ca^{2+} buffering, impaired Ca^{2+} handling and the inception of Ca^{2+}-mediated hypertrophic signaling [104]. Human HCM can be modelled also in *rats*. A transgenic rat model of HCM, expressing a truncated human TnT protein missing exon 16, was characterized by a degree of diastolic dysfunction similar to that observed in the mouse model, as well as increased predisposition to ventricular arrhythmias [105].

Besides their usefulness in assessing the patho-mechanisms and electro-mechanical abnormalities characterizing human HCM, transgenic murine models represent a fundamental basis to test novel molecules with possible pharmacological action, before proceeding with larger animal models and, eventually, with clinical trials in patients (Table 1). The R403Q-MHC model, the first animal model developed to study the pathogenesis of HCM [79] was used to test the L-type Ca^{2+} channel inhibitor **diltiazem**, which showed to prevent the development of HCM-related pathological changes in transgenic mouse hearts. In particular, decreased levels of RyR2 and SR Ca^{2+} binding proteins such as calsequestrin were observed in αMHC403/+ myocytes and were restored to normal levels as a direct consequence of the inhibition of L-type Ca^{2+} channels by diltiazem. As a consequence of the reduced intracellular Ca-overload, the early administration of diltiazem to αMHC403/+mice reduced cardiac hypertrophy and fibrosis, thus preventing the cardiac histopathology and hemodynamic consequences of this sarcomere protein mutation [106]. In line with that, preventive treatment with diltiazem of HCM mutation carrier individuals (identified in family screening programs), prior to the development of HCM cardiac phenotype, delayed the progression of diastolic dysfunction an ventricular thickening [107]. As mentioned above, I_{NaL} is deeply involved in HCM pathogenesis. R92Q-TnT transgenic mice accurately reproduce all different pathophysiological features of HCM, including the increased I_{NaL}. Acute treatment with ranolazine lowered both intracellular [Na^{+}] and diastolic [Ca^{2+}] in R92Q-TnT cardiomyocytes through the inhibition I_{NaL} [95]. We established to chronically treat R92Q transgenic mice with oral ranolazine since birth, to evaluate whether ranolazine treatment effectively blocked the development of diastolic dysfunction in HCM transgenic mice [95]. Our results showed that life-long ranolazine treatment exerted a preventive effect on the development of HCM patho-mechanisms in the hearts of R92Q-TnT murine models, counteracting the onset of LV hypertrophy, tissue fibrosis and diastolic dysfunction. In particular, the prevention of pathological excitation-contraction-coupling abnormalities is responsible of the preserved diastolic function in treated R92Q-TnT mice. In fact, in cardiomyocytes isolated from ranolazine-treated mice, the physiological diastolic Ca^{2+} level is preserved and Ca^{2+} transient kinetics is faster because of the maintained SERCA and NCX function [95].

The increased production of ROS demonstrated in the hearts of patients with HCM may mediate HCM-related electro-mechanical abnormalities in combination with intracellular Ca^{2+} overload [47]. Therefore, the use of anti-oxidant compounds could represent a helpful strategy in the therapeutic management of HCM. Moreover, considering that ROS are key determinants of the progression of cardiac hypertrophy, the oxygen radical scavenger compound **N-acetyl-cysteine** (NAC) could act a leading role in the reduction of the pathological burden in the ventricles of animal models and patients with HCM. NAC was tested on a mouse TPM1-HCM model and it effectively reduced diastolic dysfunction and hypertrophy [108]. Echocardiography was used to evaluate heart morphology and diastolic function. While treatment with NAC exerted a partial amelioration on left atrium (LA) size, it significantly reduced left ventricle (LV) mass, returning it back to NTg levels. NAC treatment was also capable to correct diastolic dysfunction and reduce E/A ratios to NTg levels [108].

As stated above, the main role played by the metabolic impairment in the pathophysiology of HCM [71] and the resulting increased energetic cost of myocyte contraction [35] suggest that reverting energy depletion could represent a major improvement in the therapeutic approach of HCM. The ability of the metabolic modulator perhexiline to shift metabolism from free fatty acids to glucose utilization in HCM patients [63] was also tested by Gehmlich and coworkers in Mybpc3-targeted knock-in mouse model of HCM [109]. Performing non-targeted metabolomic analysis (applying ultra-high performance liquid chromatography-mass spectrometry), Gehmlich and colleagues observed that perhexiline administration induced a phenotypic modification of the cardiac metabolome with 272 unique metabolites, impairing fatty acids and improving glucose utilisation, thus suggesting an evidence of altered fatty acid transport into mitochondria and increased glucose utilisation. These data suggest that perhexiline treatment significantly modify **cardiac metabolome** increasing ATP production and myocardial efficiency, thus ameliorating HCM phenotype in Mybpc3-targeted knock-transgenic model [109].

Intrinsic myofilament hyper-activation and abnormal myofilament energetics are believed to represent the primary change determined by sarcomeric HCM. As a consequence, correcting the primary energetic abnormalities in mutant myofilaments may prevent the formation of LV hypertrophy and of the arrhythmogenic tissue substrate. Myosin heads rapidity of movement along actin filaments and their ability of force generation directly influence sarcomere power output. Considering that increase of the total cycle time of myosin ATPase reduces the temporal interaction between cardiac myosin and actin, resulting in fewer myosin molecules in an active state to generate force, accordingly decreasing ensemble force generation, Green and coworkers screened different molecules for their capability to reduce the maximal actin-activated ATPase rate of myosin, and tested them in bovine myofibrils [110]. Among the chemical compounds identified through this screen, they focused on MYK-461, evaluating in-depth its potency and the pharmaceutical properties. Treatment of mouse cardiac myofibrils with the myosin inhibitor **mavacamten** (MYK-461) reduced ATPase activity in a dose-dependent manner; in particular, maximal doses of MYK-461 (>10

μM) reduced the maximal ATPase rate by ~90 %. Individual steps of the myosin chemomechanical cycle through that MYK-461 inhibits myosin ATPase were then analyzed in detail by Green and coworkers performing transient kinetic experiments: the rate of phosphate release and the rate-limiting step in the chemomechanical cycle were observed to be reduced in a dose-dependent manner by the administration of MYK-461, that exerted its effects without slowing adenosine diphosphate (ADP) release. The aforementioned data show that MYK-461 decreases the ratio of strongly bound state time to total cycle time of myosin, consequently reducing the ensemble force, power, and contractility produced by the sarcomere [110]. With this mechanism, mavacamten restored the correct myofilament energetic properties in a mouse MYH7-HCM model and prevented the development of both LV hypertrophy and fibrosis [110].

However, no significant effects on disease onset and progression were noticed when the drug was administered to a mouse MYBPC3-HCM model [111], suggesting that myosin inhibition could be a relevant therapeutic option for specific HCM genotypes.

2.2.2. Rabbit models

Notwithstanding their avail in the investigation of the molecular basis of HCM, rodent models show different limitations and therefore show constrained predictive significance in translating relative pathophysiological information to humans. These limitations, mainly driven by species differences (e.g., anatomy, physiology, metabolism, lifespan, and response to treatment), could promote translational failures when drugs that showed promising results in rodents are later tested in human clinical trials [112]. Significant differences between the mouse and human are physiologically evident in the regulation of Ca^{2+} homeostasis during contraction and relaxation and when disease-specific pathological alterations of Ca^{2+} fluxes occur [113,114]. Moreover, rodent models show limitations for their use in evaluating the increased ventricular arrhythmogenesis, one of the hallmarks of HCM. In fact, the beating typical of the mouse heart is almost ten times faster than the human heart and this faster rate influences the refractory period, which directly correlates with the incidence of arrhythmias [115]. The aforementioned limitations underscore the importance of working on relevant preclinical models that represent an intermediate step between transgenic rodent models and human clinical medicine [112]. Rabbits are unlikely to represent the primary transgenic model to study HCM because of the difficult process and wide expenses needed for developing these transgenic lines, as well as the lack of validated advanced genetics approaches in this species [116]. However, they show a number of advantages over the mouse, for studying cardiovascular disease [117] (Fig. 2). In fact, rabbits more accurately reflect the human system and are larger in size, which facilitates investigations such as echocardiography or invasive electrophysiology. Other advantages of transgenic rabbit models are related with the expression of β-MyHC: in the mouse the β-MyHC protein is expressed in the ventricles only at subsided levels [118], but represents the predominant protein in the ventricle of rabbits (the same as in humans), showing an approximately 98 % homology to human β-MyHC protein [119,120]. Moreover, skinned rabbit and human cardiomyocytes are characterized by similar contractile properties [121].

As mentioned above, the first animal model developed to gain deeper insights into the pathogenesis of HCM was the MyHC-R403Q transgenic line [28]. The demand for an animal model that more closely and faithfully recapitulated human HCM clinical/pathological phenotype was fulfilled by producing a transgenic rabbit harboring the R403Q mutation in human β-MyHC [117]. The phenotype of the R403Q transgenic rabbit virtually replicated that of humans, showing gross cardiac hypertrophy, myocyte and myofibrillar disarray, interstitial fibrosis, diastolic dysfunction associated with preserved systolic function. Moreover, these transgenic rabbits were characterized by a high occurrence of premature death. The aforementioned pathogenic features make the β-MyHC-R403Q transgenic rabbits a suitable proxy of human HCM for pathogenetic and therapeutic studies. To further evaluate the different patho-mechanisms of HCM and determine the temporal evolution of cardiac HCM phenotypes, Sherif and coworkers deeply investigated the R403Q transgenic rabbit over a four years period using multiple techniques [122]. Ca^{2+} sensitivity of myofibrillar ATPase activity decreased very early during the progression of cardiac pathology, suggesting that the impairment of myocardial energetics may represent a primary defect contributing to the pathogenesis of HCM [123]. Myocyte disarray is another HCM feature that occurred early, independently from hypertrophy and fibrosis. Finally, β-MyHC-R403Q transgenic rabbits showed a progressive deterioration of ventricular diastolic function with aging, replicating the evolution of HCM in humans [1]. Collectively, these pieces of evidence suggest that different independent mechanisms contribute to the pathogenesis of HCM [122]. Lowey and co-workers used a similar transgenic rabbit to evaluate the consequences of the R403Q mutation on actin–myosin interactions at the molecular level [124]. They observed myosin loss-of-function in mutated myofibrils from the rabbit, comparable to the findings from studies in transgenic mouse models with the same mutation [125,126]; indeed, R403Q myofibrils generated a reduced power when compared with control myofibrils. In particular, the maximum tension, as well as the kinetic rates for activation and myofibril relaxation, were all significantly reduced in the ventricular R403Q myofibrils with respect to controls. A transgenic rabbit carrying the cTnI-R146G mutation in cTnI gene was generated and studied [127]. The ventricles from the cTnI-R146G rabbits showed myofiber disarray, interstitial fibrosis and mild apical ventricular hypertrophy at 18–24 months of age. All in all, transgenic rabbit models represent a novel and informative source to investigate the HCM-related pathogenic mechanisms and develop innovative therapies [127]. The increased production of reactive oxygen species observed in human HCM cardiomyocytes led the researchers to test antioxidant compounds on transgenic rabbit models of HCM. Thus, based on the rationale that reactive oxygen species are important determinant of disease progression in cardiac hypertrophy including HCM, NAC was used to treat a rabbit MYH7-HCM model [128]. Treatment with NAC caused the reduction of established LVH and fibrosis in a rabbit MYH7-HCM model, showing the beneficial role of NAC on histopathological features of HCM. In particular, echocardiographic indices of cardiac hypertrophy were regressed to normal values after 12-month treatment with NAC. Myocyte cross-sectional area was increased by ≈20 % in transgenic rabbits in the placebo group compared with the non-transgenic group. In contrast, myocyte cross-sectional area in the NAC group was comparable to that in the nontransgenic rabbits [128]. Statins may also represent a helpful approach to reduce the fibrotic condition in hypertrophic ventricles, one of the main histopathological alterations of human HCM. Indeed, treatment with atorvastatin reduced cardiac hypertrophy and fibrosis in a rabbit HCM transgenic model [129]. Despite these promising preclinical results, a pilot clinical trial with NAC in 42 patients with HCM showed no significant effects on LV hypertrophy and fibrosis, as evaluated with cardiac magnetic resonance [130]. Moreover, a pilot study with atorvastatin in 32 HCM patients did not show any changes of the echocardiographic indices of cardiac hypertrophy and function in treated subjects [131].

2.2.3. Zebrafish

The zebrafish (*Danio rerio*) is a novel research tool to gain deeper insights into the genetic basis of human cardiomyopathies. Indeed, zebrafish models have multiple interesting advantages: 1) availability of simple gene editing approaches, 2) easy to use for drug screening, 3) low cost of breeding and maintenance, and 4) accessibility of both embryonic models and adult animals. Moreover, the cardiac anatomy of zebrafish, with a single ventricle and a single atrium, makes zebrafish one of the simplest vertebrates for the study of human cardiomyopathies [132].

Zebrafish genome sequencing was finally completed in 2013, showing that 82 % of the known human pathology-related genes have an

orthologous gene in the genome of the zebrafish. This simple animal model can be combined with the application of TALEN or the CRISPR/Cas9 genome-editing toolsets. Such techniques can be used for the introduction of specific DNA elements, such as the specific genetic variants observed in patients with cardiomyopathies, by homologous recombination, thus creating disease models carrying patient-specific mutations. Moreover, the translucency of zebrafish embryos represents a useful feature, allowing exploitation of high-performance high-resolution in vivo observations of the heart. Such advanced imaging approaches helped investigating the mechanisms and timing of cardiomyocyte differentiation and maturation during cardiac development [133].

MacRae and colleagues developed a zebrafish model of a pathogenic variant of human cardiac *TNNT2* [134]. Besides increasing the knowledge on the early developmental consequences of HCM-related mutations, this transgenic research tool led to a deeper comprehension of the genetic and environmental features prompting the onset and progression of pathological myocardial remodelling in HCM. Moreover, they demonstrated that this *TNNT2* mutation perturbed the physiological Ca^{2+} handling of the cardiac cell. McRae and coworkers performed high-resolution Ca^{2+} imaging in order to determine the effects of sarcomeric *TNNT2* mutation on cardiomyocyte Ca^{2+} handling during early heart development of Zebrafish embryos. The most evident difference between pathologic and control embryos was the shortening of the Ca^{2+} transient duration (CTD50) in the *TNNT2* mutant hearts. This shortening was observed in both atrium and ventricle but was particularly evident in the outer ventricular curvature and in the mid ventricle [134]. These results supported the idea that that the arrhythmic risk observed in HCM patients is not a simple and direct consequence of cellular disarray, but involves specific alterations of cardiomyocyte electrophysiology and Ca-homeostasis. This embryonic zebrafish model carrying a patient-specific sarcomeric mutation replicated several cellular features observed in adult animals and patients carrying these mutations. Sarcomeric disarray, one of the main pathological features of human HCM, was observed also in the early-stage embryonic cardiomyocytes. The myocardial response to sarcomeric gene mutations, in terms of gene expression changes, are quite preserved in the zebrafish, notwithstanding the phylogenetic distance between the zebrafish and other mammal models [134]. One of the limitations of the zebrafish for modelling cardiac pathologies is its high cardiac tissue regeneration rate, allowing zebrafish to fully reconstitute its heart within several days, even if the damage is quite extended in the organ. This feature limits the use of the zebrafish as an optimal model for human cardiomyopathies [135], as cardiac regeneration does not significantly occur in mammals.

2.3. Larger animal models

2.3.1. Transgenic HCM pigs

Rodent models cannot fully reproduce the human HCM phenotype, showing several limitations that limit their use in cardiovascular research and cardiomyopathy modelling [125], due to the significant differences between rodent and human cardiac electrophysiological and mechanical properties. Besides the development of new larger animal models, such as transgenic rabbits, **domestic pig** [136,137] could represent an innovative tool to study molecular basis and patho-mechanisms of human HCM. In fact, considering its high resemblance to the human body in terms of cardiac anatomy, cardiovascular function, and electrophysiology [138,139], the domestic pig represents a useful model to study cardiovascular diseases (Fig. 2). Moreover, myosin isoform expression changes during cardiac development are equivalent in human and porcine fetuses [140,141], considering that both species are characterized by a gradual increase of ß-MyHC expression levels in the ventricles, and a concurrent reduction of α-MyHC expression in the second half of gestation [140,141]. Specifically, ß-MyHC is the predominant isoform of the adult left ventricle and only 7–10 % of total ventricular myosin is α-MyHC in the adult human myocardium [142,143]; α-MyHC shows very similar expression levels in pig ventricles (about 10–12 %) [144]. In particular, considering the strong influence exerted by α- vs. ß-MyHC on the consequences of specific mutations on myosin function [125], mutations in murine α-MyHC (the predominant ventricular isoform in mice and rats) cannot precisely reproduce the functional abnormalities that are observed in the human ß-MyHC. Thus, the development of transgenic pigs carrying variants in the ventricular ß-MyHC is of utmost interest for cardiovascular research, since it will provide an informative model to evaluate their effects in animals model, faithfully replicating the cardiovascular physiology of humans.

Montag and coworkers, using a TALEN-guided approach, mutated the *MYH7* gene in the porcine genome through the insertion of the orthologous HCM-related mutation R723G. The early death observed in piglets suggested that the R723G mutation is responsible of a severe form of HCM, featuring early prenatal disease development [145]. Transgenic animals were characterized by signs of myocyte disarray and mechanical abnormalities of sarcomere function in cardiac tissue, that are likely suggestive of myofibrillar loss, one of the key features of HCM [146]. SD represents a common event among young patients carrying the same mutation (R723G into the MYH7 gene [147]). According to a recent hypothesis, many myosin missense HCM mutations cause the typical myocardial hypercontractility by shifting the equilibrium between the closed state of myosin heads (super-relaxed state) and their opened state (disordered-relaxed state, available for actin-interaction) towards the latter [148,149]. Anderson and coworkers generated R403Q minipigs carrying the heterozygous MYH7 R403Q mutation, which rapidly develop a cardiac phenotype with hypercontractility and hypertrophy, consistent with HCM. The hypercontractility displayed by this large HCM animal model was explained taking into account a consistent reduction in the percentage of super-relaxed state (SRX) of myosin in the porcine fibers. The administration of the cardiac myosin inhibitor mavacamten to the R403Q fibers from the pig model raised the percentage of the SRX in the porcine fibers by stabilizing their super-relaxed (SRX) folded state, consequently decreasing total tension measured in skinned porcine cardiac muscle fibers. In particular, mavacamten restored the percentage of SRX in R403Q porcine fibers back to the normal WT levels, suggesting an innovative way to modulate cardiac contractility at the molecular sarcomeric level [150].

2.3.2. Spontaneously-occurring HCM in cats

Feline HCM represents an excellent natural model to study the pathomechanisms of HCM and to identify potential novel targets for pharmacological therapies, given the genotypic and phenotypic similarities to the human disease [151,152], the rapid progression of the disease, and the well-defined clinical endpoints. The cat model ideally overcomes several limitations of rodent HCM models, providing an improved translation from animal studies to human clinical trials (Fig. 2). Feline HCM occurs spontaneously and with a relatively high frequently in some cat races. Disease presentation in cats is remarkably similar to that in humans [151,152], although more severe with mortality mainly driven by refractory heart failure due to severe left ventricular outflow obstruction. HCM can be identified casually in cats when a cardiac murmur is auscultated by the vet. After the diagnosis of HCM, most cats die from heart failure or sudden arrhythmic death, while a smaller part of them remains subclinical. Diagnostic tests in cats (e.g. plasma natriuretic peptide, radiography, electrocardiography, and echocardiography) are also similar to those in HCM patients, as well therapeutic interventions [112], in particular **β-blockers** and **diuretics**. Among the overall feline population, the Maine Coon and Ragdoll cats are affected by HCM with a relatively high frequency, mainly driven by inbreeding practices. Known HCM-related mutations in Maine Coon and Ragdoll cats are located in the myosin binding protein-C gene, where a very limited number of pathogenic variants accounting for all cases of HCM [112]. While thousands of genetic variants have been identified in

humans and their pathogenicity have been deeply assessed [1], genetic studies in cats are partial and limited to single variants of the most involved sarcomeric genes (two variants in MYBPC3 and one variant in MYH7). In particular, the only two genetic variants associated with feline HCM (p.A31P and p.R820W) in the MYBPC3 gene were identified with high frequency also in non-affected cats, suggesting a potential contribution of other genetic or environmental factors to the HCM phenotype, thus strengthening the doubt about their pathogenicity in heterozygotes [153]. The natural history of HCM in Maine Coon cats mimics that observed in humans and it is transmitted in an autosomal dominant way [154]. Kittleson and coworkers first described a colony of Maine Coon cats with an inherited, autosomal dominant form of HCM that mimics the human disease, replicating most of its common morphological characteristics [152]. Affected cats usually do not develop HCM before 6 months of age, overt disease develops between 6 and 12 months of life, and severe HCM progression occurs between 2 and 3 years of age. Maine Coon HCM cats show a gross asymmetric thickening of the LV wall, dynamic left ventricular outflow tract (LVOT) obstruction, disarray of cardiac fiber and fibrosis. Of note, notwithstanding all aforementioned animal models represent an efficient tool to study different patho-mechanisms of human HCM, showing histologic features of the disease (hypertrophy of cardiomyocytes, diastolic disfunction, disarray, fibrosis, and increase in mass), only cats (spontaneously developing HCM) show left ventricular outflow tract obstruction (one of the clinical hallmarks of the disease). Considering that the clinical, pathological, and inheritable characteristics of feline HCM closely resemble those of human disease, cats may play an important role for understanding the pathophysiology of human HCM and for testing novel therapeutic pharmacological approaches, to be directly translated to patients [152]. Stern and coworkers employed cats with HCM and dynamic left ventricular outflow tract (LVOT) obstruction to evaluate whether a reduction in contractility mediated by mavacamten (MYK-461) could acutely eliminate systolic anterior motion (SAM) and LVOT obstruction [155]. In an exposure-dependent manner, treatment with mavacamten selectively reduced contractility and eliminated the systolic anterior motion of the mitral valve, thus relieving LVOT pressure gradients. These results suggest that acute reduction in contractility exerted by mavacamten is sufficient to decrease LVOT obstruction, with a direct translation potential to human HCM treatment.

Mavacamten was trialed in patients with obstructive HCM. A completed open label phase 2 study showed that mavacamten effectively reduced LVOT gradients and obstructive symptoms in patients [156] with limited reduction of LV ejection fraction. These positive results were confirmed by the recently completed larger phase 3 study EXPLORER-HCM [157]. A pilot trial with mavacamten in non-obstructive patients (MAVERICK-HCM) showed a reduction of plasma pro-BNP and circulating cardiac TnI in the treatment arm [158].

Considering the paucity of data about the role of oxidative stress in the patho-mechanisms of HCM, Michalek and coworkers compared the oxidative state of cats with hypertrophic cardiomyopathy and healthy controls, focusing on the activity of specific enzymes, such as superoxide dismutase, catalase and glutathione peroxidase (GPx). Markers of increased oxidative stress were detected in feline blood serum; in particular, the activity of the scavenging enzyme superoxide dismutase was significantly reduced in the group of HCM cats. Similarly, the activity of catalase that catalyzes the breakdown of H_2O_2, was consistently lower in animals at a preclinical stage of the disease [159] (Fig. 2).

Gene transfer technology to overcome the limitations associated with transgenic animal models.

Although transgenic mice represent an effective platform to study disease-associated patho-mechanisms, the phenotype expressed in this transgenic tools is the result of a combination between primary defects directly caused by the HCM causing mutation and the consecutive adaptations provided by the animal to compensate that defect or the cardiovascular remodeling that has occurred. For example, myofibrillar disarray displayed by many of the mouse models used to study human HCM [160] could be directly caused by the HCM mutant protein but can also represent a secondary consequence of cardiovascular remodeling necessary to counterbalance functional alterations caused by HCM mutant proteins. Therefore, transgenic animal models make difficult to discriminate primary defects caused by HCM mutant proteins from compensatory changes occurring during HCM pathogenesis in vivo. The application of gene targeting and gene transfer technology to adult cardiac myocytes in mice and primary culture provided a novel approach to directly screen effects of HCM mutant contractile proteins on the structure and function of adult cardiac muscle cells, gaining insights into specific molecular mechanisms of HCM pathogenesis. Moreover, this approach allows to rapidly screen multiple mutations in contractile proteins without the cost and time related to the generation of multiple animal lines [161]. Rust and coworkers [162] described an efficient protocol that allows to transfected (through adenoviral gene transfer) adult rat cardiomyocytes to be maintained in primary culture for a period of 6–7 days, maintaining contractile protein isoform expression, stoichiometry, and force generation. As consequence, this protocol provides a stable “one-week window” in which to perform genetic manipulations of contractile proteins of the cardiac sarcomere. Moreover, Westfall and coworkers developed a tool through that adenoviral mediated gene transfer can be used to replace an endogenous protein with an expressed one. In particular, they substituted the endogenous cardiac TnI with expressed slow skeletal TnI, causing abnormalities in cardiomyocyte activity that can be directly associated to the specific manipulation of these TnI isoforms [163]. The expressed contractile protein within the cardiac muscle can then be assessed through different techniques as quantitative western blotting or high resolution immunofluorescence confocal microscopy, allowing to compare the expression of the mutant protein to endogenous proteins and the incorporation of the mutant protein in the myofilaments and its effects on sarcomere structure, respectively. However, the main limitation associated to this approach is represented by the limited possibility to translate functional measurements from single cells to the whole organ [161].

2.4. Human iPSC as an innovative approach to model cardiomyopathies

Time, cost, and species differences hamper the suitability of animal models for the study of HCM [164,106,165]. Importantly, differences in the electrophysiology and Ca^{2+} handling of cardiac myocytes significantly restrict the role of murine models in cardiovascular research [166]. The functional evaluation of genetic alterations responsible for cardiac diseases (e.g. channelopathies) has been achieved by re-expressing the mutated proteins into heterologous systems in vitro. The molecular abnormalities investigated using heterologous expression systems were essential to understand the primary pathological mechanisms of genetic diseases. However, heterologous expression systems are limited by the lack of a proper intracellular environment and the total absence of disease-associated cellular structural and functional remodeling [167]. The development of patient-derived induced pluripotent stem cells (iPSCs), which can be differentiated into functional cardiomyocytes (CMs) in vitro, may play an increasing role in the study of disease mechanisms underpinning inherited genetic heart diseases [166] (Fig. 3). Frozen iPSCs remain available for subsequent differentiation and, at variance with primary isolated CMs from adult hearts, harbor the ability to survive endlessly in culture [168]. Theoretically, any cell type can be obtained through directed differentiation from an iPSC line originated from somatic patient cells, allowing the researchers to model different inherited diseases, or to model the effects of a given mutation on different organs. iPSC-derived cells also show a huge potential for drug research and development, as they represent a relatively endless source of material where pharmacological molecule testing can be extensively performed. Moreover, iPSCs represent an unequalled platform for regenerative medical interventions [166]. In particular, iPSCs are optimal for differentiation into cells such as cardiomyocytes,

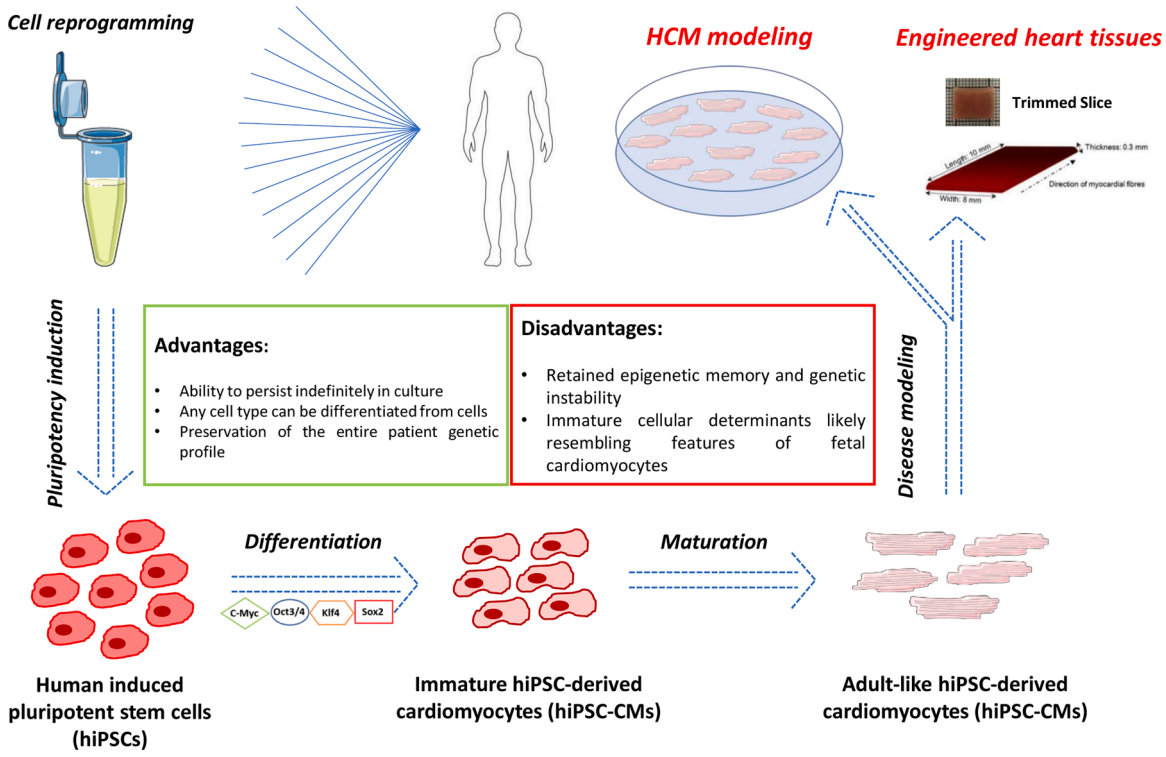

Fig. 3. IPS cells as an invaluable research tool for disease modelling and drug screening. IPS (induced pluripotent stem cells) can overcome the limitations associated with the use of human surgical samples and transgenic animal models in preclinical research, representing an innovative tool for studying specific disease patho-mechanisms and for drug discovery. Moreover, IPS cells can be employed to generate 3-D engineered heart tissues, overcoming the limitations associated to the standard two-dimensional (2-D) monolayer cell cultures, such as the possibility to efficiently measure contractile force, one of the most important parameters related to cardiac function that cannot be recorded in monolayers. Images from Pitoulis et al., 2019.

which are difficult to be isolated from patients and are characterized by almost no regenerative potential in culture [169]. Therefore, the integration of genetic analyses and modeling disease with patient-specific iPSC-derived cells holds a huge potential to improve our knowledge of the genetic causes and the mechanisms of specific diseases. Since iPSC preserve the entire patient genetic profile, the system will help scientists in identifying the most appropriate pharmacological intervention to correct specific functional alterations with an individualized, precision-medicine approach [167]. Patient derived iPSCs could play a fundamental role in preclinical drug development and safety toxicology, overcoming limitations showed by recombinant cell lines or animal models, both susceptible to several shortcomings, as discussed above. In fact, hiPSC-CM are able to well reproduce the complexities of an adult human cardiomyocyte, thus pharmacological results from studies in hiPSC-CMs can be easily translated to patients. Interpersonal variability can be easily accounted by performing drug testing in different iPSC lines with a wide spectrum of genetic backgrounds. This may also allow evaluations of the individualized disease-related risk and of the individual susceptibility to certain therapies [170]. Moreover, the application of CRISPR/Cas9 system to WT hiPSC-CMs is fundamental to gain deeper insights into disease patho-mechanisms associated to a specific mutation. In fact, the insertion of a specific mutation in WT hiPSC-CMs through a gene editing method, as CRISPR/Cas9, allows to create an isogenic control that represents the benchmark to the control line. As consequence, potential differences (as in electro-physiological parameters [171]) between control line and isogenic control can be directly correlated to the inserted mutation, thus evaluating its ability to dysregulate cardiomyocyte physiology. Considering the clear difference existing between human and rodent cardiomyocytes for what concern ion channel expression and biophysics [172], screening platforms based on WT hiPSC-CMs and suitable for medium- to high-throughput application have been recently developed to perform the early phases of drug discovery and development [173–175]. Sala and coworkers described a protocol to dissociate 2D cell cultures of hPSC-CMs to small aggregates and single cells, plating them on multi-electrode Arrays (MEA) to record their spontaneous electrical activity as field potential (FP). As consequence, perturbation of the FP waveform can be associated with changes in specific action potential phases [176], providing observations with respect to beating frequency, QT interval duration, and arrhythmic events. Compared with manual patch-clamp, MEA devices allow medium- to high-throughput recordings of the electrical waveform signals generated by monolayers or small clusters of cardiomyocytes, thus supporting the standardization of the analysis of hPSC-CM FPs and improving data reproducibility. FP traces are extracted and then used to obtain QT interval values with specific settings. In particular, analysis of QT-RR relationship allows the researcher to gain insights in the evaluation of the need and/or the effect of QT-interval corrections in diseased and WT hPSC lines, observing that hPSC-CMs carrying LQTS-causing mutations are characterized by prolonged QT intervals compared with WT controls. Moreover, while administration of a hERG activator results in shortening of the QT interval, treatment of hPSC-CMs with a hERG blocker results in QT interval prolongation [177]. This protocol developed by Sala and coworkers represents another confirmation of hiPSC-CMs efficiency in finely reproducing human pathologies in vitro, allowing specific disease-associated patho-mechanisms evaluation and drug-screening.

2.4.1. iPSC-CMs to model human cardiomyopathies: pros and cons

Despite many advantages, major limitations characterize this innovative tool, such as the difficulties in obtaining a uniform population of completely reprogrammed iPSCs [178]. To overcome this restriction, Paull and coworkers described the development of fully automated and robotic processes for generating iPSC lines of high quality and consistency, developing a modular, robotic platform for iPSc reprogramming

allowing automated, high-throughput conversion development of iPSCs and differentiated cells with negligible manual intervention. Moreover, this platform showed the ability to perform a pooled selection of polyclonal pluripotent cells, resulting in high-quality, stable iPSCs populations. In particular, Paull and coworkers analyzed enriched samples using a gene expression panel covering pluripotency and germ-layer marker genes, employing negative selection against incompletely reprogrammed cells with an immunomagnetic bead separation device (MACS) to achieve a 26-fold enrichment of reprogrammed cells, thus separating full-reprogrammed iPSCs from incompletely-reprogrammed cells. Moreover, they observed that automation of the generation of pooled, polyclonal lines slowed to remove more than one-third of the variability that existed between manually selected lines, suggesting that a consistent portion of the variation observed between manually derived iPSC lines has purely technical origins that may obscure inherent genotypic differences [178].

Briefly, cell source, genetic background and age, reprogramming procedures, culturing conditions, and many other critical factors may influence the resulting iPSCs and introduce biases [166]. However, the most consisting limitation related to the use hiPSC-CMs as a source to model cardiomyopathies is that they have immature cellular features after differentiation, which more closely resemble the functional/morphological features of fetal cardiomyocytes. Therefore, it is still controversial and debatable what stage of cardiac diseases can be effectively modelled by iPSC-CMs. Their immaturity encompasses a large spectrum of features arising from the expression of fetal genes [179,180], as well as electrophysiological signals and contractile properties that resemble fetal cells [181–183]. Moreover hiPSC-CMs are smaller in size, they show reduced electrical and contractile function, have disorganized sarcomeres and finally show an absence of t-tubules and a scarce sarcoplasmic reticulum organization [184]. Functional immaturity of iPSC-CMs was confirmed in an in vivo study, by transplanting hiPSC-CMs in a cardiac infarction model in primates [118]. The injected cells engrafted and regenerated the infarcted heart, although the issue of automaticity still remained manifesting in arrhythmogenic foci declining only over a 2–3 weeks period. Taken together, these findings suggested that maturation protocols for hiPSC-CMs are needed. In the last few years, many researchers have been exploring clues to foster maturation of hiPSC-CMs, with a wide range of methods such as growing cells on patterning scaffolds [185], or by employing 3D cell-alignment strategies [181], electrical and mechanical stimulation, co-cultures and custom growth media [186].

Among the strategies developed to improve hiPSC-CMs maturation, long-term culture on nanopatterned surfaces was described as an efficient method, producing differentiated cardiomyocytes that more closely resemble human adult ventricular cardiomyocytes, with the development of transverse (T) tubules and the expression of sarcomere protein isoforms that are indicative of the tardive stages of maturation [182,187]. Pioner and coworkers described a custom-made experimental setup for concomitant optical measurements of action potentials and calcium transients in hiPSC-CMs, which has been employed to identify a potential correlation between these parameters and specific time points of maturation (at 60, 75 and 90-day post-diff ;erentiation) in control cardiomyocytes. In particular, hiPSC-CMs were plated on hydrogel-based micropatterned substrates mimicking the extracellular matrix (stimulating cell alignment and elongation) and maintained in a long-term culture to evaluate potential developmental changes at later stages of maturation (days 60–75–90). At the tardive stages of the maturation process, single hiPSC-CMs showed prolonged action potential duration, increased calcium transient amplitude with shorter duration, characteristics that closely mirror those of human adult cardiomyocytes isolated from fresh ventricular surgical samples, demonstrating the efficiency of micropattern surfaces and long-term cultures in promoting hiPSC-CMs maturation [171]. hiPSC-CMs maturation can be promoted also by cardiac-tissue-engineering approaches, organizing immature hiPSC-CMs into a 3D environment closely resembling the physiological cardiac tissue, in combination with cardiac fibroblasts [188]. Engineered heart tissues (EHTs) can be developed by casting myocytes and non-myocytes cardiac cells into a collagen hydrogel, where cardiomyocytes can be electrically and mechanically stimulated, thus improving their maturation. Vunjak-Novakovic and colleagues developed human EHTs bearing mature structural and functional properties, despite spontaneously beating, by incorporating a cell mixture composed of human dermal fibroblasts and early-stage hiPSC-CMs into an appropriately shaped fibrin hydrogel cast and then electrically stimulating the system for three weeks at increasing frequencies (from 2 Hz to 6 Hz) [189]. The improved maturation level involved also the electrophysiological features, with mature EHTs showing a more negatively polarized resting membrane potential and an increased inward-rectifier potassium current density. Finally, EHTs developed by Vunjak-Novakovic and colleagues exhibited inotropic and lusitropic responses to β -adrenergic stimulation comparable to those observed in mature myocardium, confirming the efficiency of the method to generate mature human EHTs [189].

2.4.2. Pathophysiological and pharmacological insights from hiPSC-CM studies

In the last years, various preclinical studies have described intracellular Ca^{2+} handling abnormalities as the main determinants of electro-mechanical dysfunction in HCM. These observations are related to human ventricular cardiomyocytes isolated from human samples [47] or transgenic animal models. The central role of Ca^{2+} handling anomalies in the pathogenesis of HCM was confirmed by Lan et al., who generated functional patient-specific hiPSC-CMs from a ten-member family cohort harboring a HCM missense mutation (Arg663His) in the MYH7 gene. Besides replicating multiple features of the HCM phenotype, including cellular hypertrophy and arrhythmogenesis, pathological patient-specific iPSC-CMs confirmed that these phenotypic abnormalities were preceded by abnormalities in Ca^{2+} transients kinetics and increase of diastolic $[Ca^{2+}]_I$, efficiently elucidating that the dysregulation of Ca^{2+} cycling plays a main role as a cause of diastolic dysfunction and arrhythmogenesis in HCM [169]. These observations confirm the efficiency of iPSC technologies as novel methods to evaluate the connection between sarcomeric mutations and the development of overt HCM. hiPSC-CMs harboring the Arg663His mutation generated by Lan and coworkers replicated numerous features of the HCM phenotype in vitro: therefore, this innovative tool was also used as a screening platform to test the potential efficacy of a number of drugs at the single-cell level. Lan et al. exposed control and HCM iPSC-CMs to verapamil, a L-type Ca^{2+} channel blocker, and assessed whether pharmacological reduction of Ca^{2+} entry prevented the onset of HCM-related phenotypic changes at cardiomyocyte level. Indeed, long-term exposure of pathological iPSC-CMs to verapamil markedly reduced all aspects of the HCM phenotype including Ca^{2+}-handling abnormalities, arrhythmogenicity and hypertrophy. Similar effects were obtained with nifedipine and diltiazem, confirming that the observed effects were specific to Ca^{2+} channel inhibition. Extensive screening of other agents – currently employed to treat HCM – has also proved effectiveness: antiarrhythmic drugs that affect Na^{+} influx, such as ranolazine, lidocaine and mexiletine rescued normal beating in HCM iPSC-CMs counteracting the abnormal function of Na^{+}/Ca^{2+} exchanger and limiting the influx of Ca^{2+} into the cardiomyocyte [169].

Calcium channel blockers (CCBs) such as diltiazem and verapamil are commonly used to treat HCM patients, even with life-long treatment (Table 1). Wu and collaborators tested the long-term effects of CCBs on human iPSC-CMs in terms of transcriptome changes [190]. After 14 days of treatment a transcriptomic approach was employed to assess the genes that were up- or down-regulated by CCBs administration. Verapamil, more than the other CCBs, downregulated cardiac contraction-related genes, as well as myofibril and sarcomere structure-related pathways. The downregulation of myofilament genes in HCM hearts may, at least in part, explain the efficacy of verapamil in

managing obstructive HCM.

Another advantage of hiPSCs is the possibility to use genome-editing technologies to rapidly generate knock-out, knock-in, or reporter cell lines. CRISPR/Cas9 and TALENs allow introducing/removing specific mutations into the iPSCs genome [191,192] thus enabling the development of isogenic controls. These approaches efficiently overcome the limitations related with the use of healthy mutation-negative family members as control subjects. Isogenic controls allow researchers to directly correlate the genetic defect with any observed phenotypic variations. As a consequence, the possibility to manipulate hiPSC lines through genome editing methods may play a significant role in the comprehension of specific patho-mechanisms of monogenic diseases such as HCM [193]. Although Troponin T mutations account for about 6–8 % of patients with HCM [194], we demonstrated that Troponin T mutant mice represent excellent tools to study the pathophysiological mechanisms of human HCM, replicating many of the different phenotypes that characterize human pathology [78]. Indeed, echocardiographic measurements performed in WT, R92Q, and E163R revealed that both pathologic mice are characterized by a significantly increased septal thickness compared with WT mice, highlighting the presence of asymmetric left ventricular hypertrophy (LVH). Doppler studies of transmitral blood flow velocity demonstrated that in both mutant mice (more severely in R92Q), early LV filling was reduced and isovolumic LV relaxation time was prolonged, suggesting an impaired diastolic function. We then measured isometric force from intact left and right ventricular trabeculae, observing that both E163R and R92Q trabeculae are characterized by prolonged twitch duration compared with WT [78]. To deeper analyze the E—C coupling process, intracellular Ca^{2+} measurements were performed in isolated cardiomyocytes, showing that R92Q cardiomyocytes are characterized by a markedly prolonged intracellular Ca^{2+} transient decay compared with both WT and E163R cardiomyocytes, at all stimulation frequencies tested. Diastolic $[Ca^{2+}]_i$ was increased in both mutants but the largest change occurred in the R92Q model [78]. We then observed that both transgenic lines show arrhythmogenic activity compared to WT models at baseline, and this tendency to spontaneous events increased after the administration of isoproterenol. Western blot studies revealed that both transgenic hearts were characterized by an increased level of CaMKII autophosphorylation and this augmentation was particularly evident in R92Q hearts compared with WT mice. We finally noticed an increased amount of intramyocardial fibrosis performing Picrosirius red staining on LV tissue sections in both R92Q and E163R hearts [78].

Wang and coworkers introduced the TnT-I79N [195] mutation into hiPSCs using CRISPR/Cas9, observing that this pathological line showed myofibril disarray and increased arrhythmogenic activity, thus confirming that the pro-arrhythmic AP changes are a direct consequence of HCM-linked TnT mutations in human CMs. In particular, AP triangulation mediated by increased cytosolic Ca^{2+} binding was identified as a new mechanism of arrhythmogenesis [196]. The efficiency of genome-editing methods applied to hiPSC-CMs was confirmed also by Mosqueira et al., who used CRISPR/Cas9 editing to produce 11 variants of the HCM-causing mutation c.C9123T-MYH7 in three independent hiPSC lines [197]. The functional analysis of these lines showed a clear association between mutational load and the level of phenotypic and functional perturbation. Moreover, the effects of the mutations on mitochondrial function and on the transcriptome support the energy depletion theory of HCM pathogenesis, that is, the inefficient ATP utilization by the disorganized sarcomeres increases the energetic demands from the cardiomyocyte [197]. The ability of hiPSC-CMs to efficiently reproduce the pathogenic feature of HCM was confirmed also by Toepfer and coworkers. They developed a MatLab algorithm called SarcTrack optimized for hiPSC-CMs, efficiently evaluating the mechanics of contraction at the level of the single sarcomere by monitoring different sarcomere parameters as sarcomere count and dynamic changes in sarcomere lenght (SL). In particular, SarcTrack analysis of hiPSC-CMs carrying the *MYBPC3* mutation recapitulated the typical HCM phenotype, including cardiac hypercontractility and diminished relaxation. Moreover, through this algorithm, the application of the myosin allosteric modulator mavacamten (MYK-461) to mutant hiPSC-CMs abated the aforementioned pathological features, evidencing the potential therapeutic efficacy of this molecule in human HCM patients [198]. The potential therapeutic effect of MYK-461 in HCM patients was analysed in greater depth by the same group in a more recent work, where restoration of the appropriate myosin super-relaxed state (SRX) balance by MYK-461 led to the normalization of the biophysical and metabolic abnormalities observed in mutant hiPSC-CMs. In this work, Toepfer and coworkers studied pathogenic *MYH7* HCM mutations using patient-specific hiPSC-CMs, revealing that myosin conformations are central regulators of cardiomyocyte metabolism, balancing muscle work and metabolic costs. In fact, the alteration of myosin conformational state's balance by myosin mutations has been noticed to play an important role for the direct perturbation in sarcomere efficiency and cellular homeostasis, resulting in increased contractility at the expense of higher energetic demands and impaired relaxation. Therefore, the restoration of the physiological myosin conformational state exerted by MYK-461 could act a relevant role in the therapeutic management of HCM [199].

2.5. Engineered heart tissues techniques to overcome the limitations of 2D systems in HCM modeling

In vitro models of the heart are necessary to gain deeper insights into the mechanical and electrophysiological function of the organ in physiological and pathological conditions. Moreover, they represent an efficient platform to screen the safety and efficacy of potential novel pharmaceutical molecules. Among the different tools used to study the heart in depth [200], cell culture is fundamental to understand multiple mechanisms responsible for cell behavior in vivo, uncovering different biomolecular processes by which cells assemble into functional tissues and organs and how this function can be altered in disease condition. In particular, two-dimensional (2D) conventional cell cultures, where cells adhere to a flat surface (typically represented by a petri dish of glass or polystyrene) providing mechanical support for the cells, have represented for a long time the most used cell culture system to maintain cells [201]. In fact, the ability of 2D systems to allows cells receiving a comparable amount of nutrients and growth factors present in the medium, thus leading a homogenous growth and proliferation, makes 2D platforms attractive to biologists. However, under some circumstances 2D methods cannot control the development of important features of the cell as the cell shape (determining biophysical cues affecting cell bioactivities in vivo), thus failing to faithfully reproduce as the cell development processes observed in the physiological environment in vivo as the associated cell bioactivities [201]. Nothwithstanding the various benefits related to their use in cardiovascular research, hiPSC-CMs are often used in single-cell assays, thus showing several limitations in the capability of representing in vivo cardiac environment. In particular, single cardiomyocytes lack relevant physiological features such as cell-to-cell interactions, the extracellular mileau and the tridimensional tissue organization. Moreover, single hiPSC-CMs reveal immature functional characteristics such as the reduced sarcomeric organization of myofilaments, one of the main features of mature cardiomyocytes. Bidimensional systems cannot generate contractile force, one of the most important parameters related to cardiac function; as a consequence, cell shortening is often used as a proxy of contractility. 3D culture systems can overcome these limitations. In physiological conditions, a highly elaborate 3D microenvironment directly coordinate cell bioactivities through different signals [202,203]. Moreover, essential cellular behaviors are affected by the distribution of cell-ECM and cell-cell interactions [204]. The development of 3D cell culture platforms able to precisely replicate the complex cellular microenvironment opened the possibility to finely reproduce biochemical and biomechanical signals regulating cells bioactivities in vivo, thus accurately

modeling the in vivo interactions of tissues and organs [205]. In fact, 3D cell cultures are consistently different from standard 2D cultures for what concern cell-cell interaction, cellular mechanics, and nutrient access, mimicking with high accuracy the in vivo environment and thus promoting proliferation, migration, matrix production, and stem cell differentiation [204]. The development of 3D culture systems is closely associated to the novel platform of engineered 3D heart tissues, overcoming limitations proper of 2D tools [206] (Fig. 3). Tissue engineering represents a novel approach able to accurately replicate the myocardial niche, closely mimicking cell-cell and cell-matrix interaction and mechanics, thus improving the possibility of "in vitro" HCM modelling (Fig. 3). In particular, tissue engineering allows the generation of surrogate three-dimensional (3-D) tissues, able to reproduce physiological organ function better than the classic two-dimensional (2-D) monolayer cell cultures [207]. The "traditional" tissue engineering approach consists in building functional 3-D artificial heart tissues that can be placed onto the injured heart, such as the production of tissue matrixes that emulate the natural extracellular environment, consequently allowing cells to integrate into it and form an artificial tissue. The ability of engineered heart tissue to more faithfully reproduce human cardiac tissue than 2-D monolayer cell cultures can be explained considering that 3-D tissue allows communications of cells, creating contacts with surrounding cells in all directions, while cardiomyocytes cultured in monolayer cell cultures are plated on a rigid plastic surface that allows only side-to-side contacts with neighboring cells. Moreover, the 3-D environment stimulate differentiation of cardiac myocytes toward a completely mature phenotype, as detailed above, representing an important advantage for hiPSC-CMs differentiation [166,167]. Vunjak-Novakovic and colleagues developed a tool based on the pre-treatment of synthetic elastomeric scaffolds with cardiac fibroblasts (CFs), assuming that an environment of this kind could be helpful for cardiomyocyte, promoting their attachment, differentiation, and contractility, thereby stimulating the functional assembly of the engineered cardiac constructs. Cells isolated from neonatal rat ventricles were prepared to form three distinct populations: rapidly plating cells identified as CFs, slowly plating cells identified as CMs, and unseparated initial population of cells (US). The cell fractions were seeded into polyglycerol sebacate scaffolds using Matrigel™, separately (CM or CF), simultaneously (US), or sequentially (CF pre-treatment followed by CM culture, CF + CM) and cultured in spinner flasks. The CF + CM group was characterized by the highest amplitude of contraction and the lowest excitation threshold (ET), superior DNA content that contributed to the ET decrease, and higher glucose consumption rate (an index of metabolic activity), suggesting that presence of CF and their sequent application in cell co-culture improved the structural and contractile properties of engineered cardiac tissue. Moreover, cardiomyocytes belonging to CF + CM group (expressing cardiac markers as troponin I and sarcomeric α-actin) showed a parallel distribution, resembling organized architecture proper of the native heart, thereby enabling cells to a synchronous and vigorous contractile response. These data suggest that the co-culture of fibroblasts and cardiomyocytes improved the properties of the engineered heart tissue [189].

Many of the novel platforms developed to simulate the 3-D structure of human myocardium depend on the modulation of mechanical signals, facilitating the distribution and the alignment of cultured cardiomyocytes in the framework of an exogenous extracellular matrix. In particular, cultured cardiomyocytes are embedded in a scaffolding matrix (made of synthetic or natural polymeric materials) located within a structured platform with a well-defined shape, in order to correctly drive the structural development of the forming tissue [184,208]. While biological scaffolds do replicate more accurately the native cardiac extra-cellular matrix (ECM), synthetic scaffolds lead to more reproducible results, exhibiting stronger and more homogeneous mechanical properties. Among biological polymeric materials, liquid hydrogels such as collagen I [209–211] or matrigel [212] are often used [213] to form extracellular matrices in which freshly isolated cardiac cells will form appropriate cell-to-cell contacts and produce extracellular matrix on their own, consequently forming a mature-like tissue. These hydrogels also enact a protective role on isolated cells, preserving them from anoikis (i.e. cell death due to loss of cell-cell contacts) [214,143]. However, the main limitations associated to the use of engineered heart tissues (EHT) as tools to study HCM is that their force development is consistently smaller than human ventricular tissues [215]. Besides the application of EHT to disease modeling field, tissue engineering approach also represents a major improvement for preclinical drug development and safety toxicology. In fact, EHT allows researchers to evaluate the effects of drugs on all the main parameters of heart function, such as pace-making activity, force development, contraction and relaxation kinetics, faithfully replicating cardiac physiology.

Breckwoldt and coworkers developed a protocol to generate fibrin-based EHTs featured by a great resemblance to human heart tissue. In particular, hiPSC-CMs incorporated in the EHTs are characterized by electrophysiological properties closely similar to those of human adult CMs, representing a clear advantage over 2D systems [206]. Mannhardt and coworkers developed a hiPSC-EHT model, evaluating the morphology and function of engineered 3D heart muscle strips and their suitability for drug screening, particularly focusing on the contractile force of hiPSC-CMs within the hiPSC-EHTs. hiPSC-CM in EHT format demonstrated a high degree of similarity with native human heart tissue. Indeed, they observed that the EHT format supports excellent heart tissue formation and promotes an efficient morphological maturation of hiPSC-CMs [170]. Eschenhagen and colleagues described the generation of 3D force-generating engineered heart tissues from hiPSC-CM, evaluating their physiological and pharmacological properties. hiPSC-CMs in EHTs showed well-developed sarcomeric organization and alignment, demonstrating a high degree of similarity between hiPSC-CM in EHT format and native human heart tissue, highlighting their informative role in preclinical drug screening and disease modeling. In particular, while cardiomyocytes cultured in 2D systems are characterized by poor sarcomeric organization and less cellular alignment, cardiomyocytes in EHTs showed a highly organized sarcomere structure, starting to beat spontaneously 10–14 days after casting. Video-optical recording was performed to investigate pacemaker mechanisms in hiPSC-EHTs, showing that five different compounds (ivabradine, ryanodine, SEA-0400, isoprenaline, TTX) affected the membrane clock in hiPSC-EHTs increasing or decreasing the beating rate, thereby demonstrating the ability of hiPSC-CMs in 3D force-generating engineered heart tissues to react to pharmaceutical stimulations, faithfully reproducing the responses of native human heart tissue. Moreover, hiPSC-EHTs were tested for response to positive and negative inotropic modulators (Ca^{2+}, ouabain, isoprenaline, ryanodine, verapamil) under rate control (1–2 Hz), showing significant inotropic modifications for all compounds evaluated: while Ca^{2+} and verapamil regulated force without affecting contraction kinetics, isoprenaline mediated an increase in force development associated to characteristic positive lusitropic effect. Finally, Ryanodine showed biphasic responses typical of the native human heart, reducing or increasing force development at low (0.3 mM) and high (10 mM) concentrations respectively [170].

Such an approach aimed at disease modeling was confirmed also by Cashman and coworkers, who developed the first 3D functional hECT model of HCM, using iPSC-hECTs from BRAF-mutant cells collected from a patient with cardiofacio-cutaneous syndrome (CFCS) and evidence of HCM. Mutant engineered tissues showed increased cardiomyocyte size, higher increased contraction and relaxation rates associated with an evident arrhythmogenic substrate [216]. Among the different approaches to produce 3D EHTs, the *"CardioSlice"* method deserves a particular mention. By developing this method, Valls-Margarit and coworkers created innovative cardiac macro-tissues from hiPSCs. Specifically, hiPSC-derived cardiomyocytes and human fibroblasts were placed into large 3D porous scaffolds and were subjected to a constant electrical pacing for 2 weeks in culture, thus promoting the emergence of myocardial tissue-like properties. Continuous

electrical stimulation markedly improved electromechanical coupling, enhancing structural and functional maturation of cardiac constructs at the tissue level but resulting in minor improvements in cardiomyocyte maturation. In fact, focusing on single cell level, cardiomyocytes laying within these scaffolds showed evident features of improved cell maturation compared with cardiomyocytes cultured under 2D conditions, though features of immaturity were still present, such as the expression of a fetal-like ion channel profile [217] (Fig. 3).

3. Conclusions and development

This review summarizes the different available platforms used in the recent years to gain insights into the patho-mechanisms of HCM and to screen novel pharmaceutical drugs before they undergo clinical trials. Although human surgical samples show a very high translational value, they are characterized by several limitations, such as the scarce availability of surgical material and the wide genetic heterogeneity among patients [78]. Moreover, surgical human tissue models are representative of a tardive stage of HCM, thus making it difficult to discern between mutation-related induced primary mechanisms and secondary and tertiary mechanisms caused by myocardial adverse remodeling and disease progression. To overcome restrictions associated to the use of surgical human samples into basic research and drug screening, different types of animal models have been developed through the years, with inbred strains overwhelming genetic background differences and extensive breeding capacity guaranteeing a wide availability of vital organs to be deeply examined. Transgenic mouse models represent the starting platform to gain insights into the molecular basis of HCM, making it possible to explore different patho-mechanisms of the disease and then providing deeper comprehension into the potential novel pharmaceutical strategies for the prevention or regression of HCM. However, various limitations of rodent models, mainly due to species differences (e.g., anatomy, physiology, metabolic rate, lifespan, and response to treatment) can limit the predictive value of transgenic animals in translating novel knowledge to humans [112]. The aforementioned restrictions suggested the need to develop larger preclinical models closer resembling human HCM pathophysiology [112]. Transgenic rabbits were used as models to study HCM [116] for their ability to accurately reflect the human system combined with their larger size as compared with mice. However, among larger animal models, pigs and feline surely play a leading role for pre-clinical research. In fact, given the genotypic and phenotypic similarities to humans, including cardiac anatomy, cardiovascular function, and electrophysiology [136–139,151,152], feline and pigs represent a novel and informative platform to study the patho-mechanisms of human HCM and thus to test novel molecules. In particular, feline HCM represents an excellent natural model to study the patho-mechanisms of the disease and play an important role for drug screening. The restrictions affecting the use of transgenic models in translational research and pharmacological testing, such as time, cost and species differences [164,106,165] can be surmounted by parallel implementation of patient-derived hiPSC-CMs. This innovative tool, supplying a potentially unlimited amount of human cells preserving the entire genome of the patient, may represent an exciting new approach for modelling inherited heart diseases as HCM [166] and an important way to screen and test novel drugs for HCM therapy.

Application of gene editing approaches as CRISPR/Cas9 system to WT hiPSC-CMs is fundamental to analyze in detail disease patho-mechanisms associated to a specific mutation. In fact, the insertion of a specific mutation in WT hiPSC-CMs through CRISPR/Cas9 results in an isogenic positive control, allowing to directly correlate potential differences (as in electro-physiological parameters) between control line and isogenic control to the inserted mutation [171], thus evaluating its ability to affect cardiomyocyte physiology. In vitro gene editing approaches may also be used as a proof of concept for in vivo therapies aimed to “cure”, rather than alleviate, HCM symptoms and risks. Indeed, current pharmacological strategies for HCM cited in this review can weaken disease-associated symptoms, slowing down disease progression, but cannot durably correct the genetic, hereditary defect at the base of HCM. Among the different available tools (antisense oligonucleotides, RNA interference molecules or wild-type cDNA sequences) able to edit DNA in gene therapy approaches, the most used is represented by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, often combined with Adeno-associated virus (AAV) delivery. The ability to transduce terminally differentiated cells and long-lasting gene expression makes the use of AAV attractive for gene therapy [218]. Many reports recently described the application of CRISPR/Cas9 system to hiPSC technique, reporting favorably creation of HCM models or genetic correction in HCM hiPSC [196,197,219]. In particular, Jehuda and coworkers [219] described the correction of the PRKAG2 gene mutation through CRISPR/Cas9 technology in the iPSC-CMs from a patient affected by HCM. Comparing the patient’s iPSC-CMs and the resulting isogenic control created by the application of CRISPR/Cas9 technology, the authors observed that the electro-physiological (delayed afterdepolarizations, triggered arrhythmias, and augmented beat rate variability) and structural (cardiomyocyte hypertrophy) abnormalities exhibited by *PRKAG2*-mutated iPSC-CMs were abolished, suggesting an efficient correction of the genetic defect responsible of HCM exerted by CRISPR/Cas9 system in the patient’s iPSC-CMs [219]. Another sector where CRISPR/Cas9 genome editing technique found both acclaim and concern is represented by the human germline therapy, since different reports described efficient application of CRISPR/Cas9 to human embryos [220–222]. For instance, Ma and coworkers reported the successful correction of germline mutations mediated by the activation of germline-specific DNA repair response in male patient with a familial history of HCM caused by a deletion in the MYBPC3 gene [221].

According to a recent report [223], telomere shortening may represent a hallmark of genetically induced cardiomyopathies, such as HCM. Sharifi-Sanjani and coworkers investigated telomere length (TL) in cardiomyocytes isolated from human cardiac tissues procured from 2 separate patient groups: 37 patients with end-stage HF transplant (including 17 HCM patients) and 26 nonfailing donors with no history of HF (NFDs). In particular, the authors observed that TL is significantly shorter in HCM hearts compared to healthy individuals, promoting TL as a specific feature oh HCM cardiomyocytes. Moreover, cells isolated from patients with reduced ejection fraction showed the shortest telomeric lengths, suggesting a potential link between ejection fraction and the severity of the disease [223]. Considering the abnormal TL observed in HCM cardiomyocytes, novel therapeutic strategies could be based on drugs/tools able to restore the physiological telomere length, thus potentially ameliorating abnormalites associated to HCM mutations. The impressive progression in these fields opens open new horizons for HCM therapies.

Declaration of competing interest

The authors have no competing financial interests to disclose.

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