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Authors	Corrado Poggesi (UNIFI)
Contributors	Michael Regnier (UW), Michael Geeves (UNIKENT)
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Executive summary

Familial CardioMyopathies (FCM), in particular Hypertrophic and Dilated CardioMyopathies (HCM and DCM), are considered diseases of the sarcomere because, they are often associated to mutations of proteins of the sarcomere, the contractile machine of the heart. We need to know the mechanisms by which mutations affect protein behaviour and hence cardiac contraction, and how adaptation to these changes results in cardiac disease. If we can understand the problem, we can better identify or design small molecules that can be used to correct the defect and prevent or manage the disease. For example, do all HCM-linked mutations cause the same cell response that leads to the disease or is there a different set of responses i.e. multiple forms of HCM that will need differently designed treatments? Currently we do not know enough about the molecular details to model the cardiac muscle system effectively.

Few mutations have been thoroughly explored at the protein, cell, organ and patient level. Therefore, a major goal of WP2 (both task 2.1 and 2.2) has been to explore mutations in the cardiac system at the molecular, cellular, and tissue level, to build a repository of data that is fed into appropriately detailed models (with WP5) that can validate the models and become a useful tool for predicting the impact of novel mutations and exploring how small molecules may provide potential new therapeutics.

The division between D2.1.(Proteins and Cells) and D2.2 (Cells and Tissue) is arbitrary and the two tasks have worked very closely together throughout with free exchange on information and experience. The reports reflect this overlap. To make clear the advantages arising from this intimate synergy, specific aspects of each report (in italics) are duplicated where relevant.

Performing task 2.2 required access to the normal cardiac myofibrils/cells/tissue and the cardiac myofibrils/cells/tissue carrying one of the many diseases linked mutations. We have obtained these using hearts from animal models and human cardiac tissue that includes patient surgical tissue samples, human tissue from donor or transplanted hearts and patient blood samples that are sources to create adult stem cells that can be turning into patient-specific heart muscle cells.

The following has been achieved:

1- Physiological measures in transgenic mice expressing engineered cardiac troponin C mutations with increased and decreased response to calcium, demonstrate phenotypes consistent with HCM and DCM diseases. The result suggests that the altered calcium responsiveness can drive HCM and DCM initiation and progression.
2- Sarcomere mechanics, kinetics, and energetics have been investigated in preparations of human cardiac samples from donor hearts and HCM patients expressing specific HCM mutations in different sarcomere proteins. The results suggest that excess/inefficient ATP utilization by myofilaments plays a central role in the pathogenesis of HCM. This disease mechanism must be taken into account by both therapeutic strategies and cardiac modelling work.
3- The impact of three small molecules (Mavacamten, Omeocamtiv, and 2dATP) that directly target myosin, the molecular motor of the heart, has been tested *in vitro* in the cardiac muscle of human and animal models. The MUSICO platform is used to simulate and predict the effects of at least some of these small molecules at multiple scales, from molecules to whole ventricle.
4- Secondary alterations of the electro-mechanical and Ca^{2+} cycling profile of cardiomyocytes from HCM patients have been investigated. The results have been used to suggest how to enrich the pharmacological armamentarium to control HCM. Future work in WP5 will include modelling of action potentials, Ca^{2+} cycling, and twitch contractions of normal and HCM human cardiac muscle and predictions of the impact of drugs.
5- In UW blood samples from UNIFI heart HCM patients carrying the same specific mutation in Myosin Binding Protein C (MyBP-C) were induced into becoming Pluripotent Stem Cells (iPSC),

then converted into heart muscle cells in cell culture. These cells carry the same mutation as the patient and allowed a study of protein expression levels, electrophysiology, Ca^{2+} cycling, and mechanics of contration. iPSCs containing the mutation in MyBPC were successfully grown and characterised; they will also be used to test potentially useful drugs.

6- Using experimental data from both intact cardiomyocytes and heart tissue we have used MUSICO to simulate the contraction relaxation cycle of normal cardiac muscle of rats and humans and of mouse cardiac muscle carrying mutations in troponin. The work demonstrates that detailed knowledge of the functional impact of sarcomeric protein mutations can be used to predict the behaviour of muscle cells.

The work described in both D2.1 and D2.2 paves the way for wide ranging studies to define how specific mutations alter the contraction of heart muscle cells and changes the performance of the whole heart. It also allows potential therapeutic drugs to be designed and tested and to then make predictions as to how these may correct the problem at the whole heart level.

Table of Contents

1. Introduction 9
1.1 The problem of modelling Genetic CardioMyopathies. 9
1.2 Potential drugs targeting the molecular motor of the heart. 9
1.3 Alterations in Excitation-Contraction Coupling in HCM and additional therapeutic targets. 10
1.4 Induced Pluripotent Stem Cells from human patients with FCM. 10
1.5 Collaboration with WP5 MUSICO. 11
1.6 Published outputs 12
2. Results from Physiological Experiments 13
2.1 Studies of transgenic mice with HCM- and DCM-like mutations. 13
2.2 Data collection from myofibrils isolated from human HCM samples about the impact of specific mutations on sarcomere function. 17
2.3 Effects of small molecules acting on myosin motor function that are good candidates for cardiomyopathy therapy. 21
2.3.1 Mavacamten, a small molecule that may improve cardiac function in HCM. 22
2.3.2 Omecamtiv, a small molecule that may improve cardiac function in DCM. 23
2.3.3 2' deoxy-ATP (dATP), a small molecule that enhances cardiac function in heart failure. 24
2.4 Data collection from intact cardiac preparations from human HCM samples about the impact of the disease on Excitation-Contraction Coupling and the use of negative inotropic drugs. 25
2.5 Human inducible pluripotent stem cells (hiPSCs) from patients with sarcomere protein cardiomyopathy mutations as a model system to investigate the impact of the disease at early stages on both sarcomere and excitation-contraction coupling functions. 27
2.6 Collaboration with WP5: Multiscale modeling of twitch contractions from human and animal models: impact of cardiomyopathy mutations and small molecules acting on the myosin motor. . 29
2.6.1 Multiscale Modeling of Twitch Contractions in Cardiac Trabeculae. 29
2.6.2 The Effect of Variable Troponin C Mutation Incorporation in Thin Filaments on Cardiac Muscle Twitch Contractions. 30
2.6.3 Next Steps. 30
3. Deviation from the work plan 31
4. Conclusions 32
4.1 Summary of achievements and key conclusions. 32
5.2 Planned future work. 32
5.2.1 Complete publications. 32
5.2.2 Further studies and plans related to SILICOFCM 32
5. References 34

6. Appendix 1. Publications arising from WP2 supported by SILICOFCM 38

List of Figures

Figure 1. Relationship of WP2 (blue /black boxes) with the broader SILICOFCM project and the modelling packages of WP5 (yellow/green boxes). 11
Figure 2. Effect of temperature on Ca^{2+} transient and twitch force in rat ventricular trabecula under fixed-end isometric conditions: experimental tracings versus MUSICO model predictions. 14
Figure 3. Twitch contractions for WT, I61Q and L48Q trabeculae from transgenic mice (TgMouse) compared to MUSICO predictions. 14
Figure 4. Model predicted impact of mutant protein penetrance on peak twitch tension. 15
Figure 5. The effect of clustering of mutated TnC's in each actin filament. 16
Figure 6. Methods to measure human cardiac myofibril mechanics & kinetics and human cardiac trabecula mechanics & energetics from patient samples. 17
Figure 7. Prevalence of the c.772G>A (E258K) mutation in MYBPC3 in the Florence HCM cohort and the demonstration of a founder effect. 19
Figure 8. Impact of E258K *MYBPC3* mutation on myofibril mechanics and kinetics. 20
Figure 9. Impact of the E258K mutation on sarcomere energetics. 21
Figure 10. Mavacamten (MAVA) decreases maximal Ca^{2+} activated force of skeletal and cardiac myofibrils. 22
Figure 11. Dose dependent effects of Omeocamtic Mecarbil (OM) on Ca^{2+}-activated tension and rate of tension redevelopment (k_{TR}) of human donor ventricular myofibrils. 24
Figure 12. Effects of Disopyramide in HCM Cardiomyocytes. 26
Figure 13. In vitro human heart muscle cell models from iPSCs of E258K MyBP-C HCM patients of the UNIFI cohort. 28
Figure 14. E258K mutation in *MYBPBC3* causes cMyBP-C haploinsufficiency in both the patient samples and in the iPSC-Cardiomyocyte *in vitro* model of the disease. 28

List of Tables

Table 1. Mechanical and kinetic parameters of myofibrils from the homozygous K280N cTnT HCM patient and different groups of control patients (top 4 rows of data) and impact of cTn exchange in K280N and donor myofibrils (bottom 6 rows of data). 18

List of Abbreviations

Abbreviation	Explanation
cTnC	cardiac Troponin C
cMyBP-C	cardiac Myosin Binding Protein C
dATP	2’ deoxy Adenosine Tri Phosphate
DCM	Dilated CardioMyopathy
Diso	Disopyramide
FCM	Familial CardioMyopathy
HCM	Hypertrophic CardioMyopathy
hiPSC	Human inducible Pluripotent Stem Cells
MAVA or Mava	Mavacamten (MYK-461)
MBPC3	Gene expressing Myosin Binding Protein C
MyBP-C	Myosin Binding Protein C
OM	Omeocamtiv Mercabil
PBMCs	Peripheral Blood Mononuclear Cells
SRX	Super Relaxed Crossbridge State
Tn	Troponin
Tm	Tropomyosin
WT	Wild Type

1. Introduction

1.1 The problem of modelling Genetic CardioMyopathies.

Most Familial CardioMyopathies (FCM), in particular Hypertrophic CardioMyopathy (HCM) and Dilated CardioMyopathy (DCM), are caused by inherited mutations in sarcomeric proteins: Myosin, Troponin (Tn), Tropomyosin (Tm), Myosin Binding Protein-C (MyBP-C) etc. [1,2]. To identify small molecules that can be used to prevent or manage the disease, we need to know the mechanisms by which each mutation affect protein behaviour and hence cardiac muscle contraction. A major goal of WP2 has been to explore the mutations in the cardiac system at the molecular, cellular, and tissue level, to build a repository of data that is fed into appropriately detailed models (described in WP5), that can become a useful tool for predicting the impact of novel mutations. Building a repository of experimental data required access to the normal healthy cardiac myofibrils/cells/tissue and the cardiac myofibrils/cells/tissue carrying one of the many disease-linked mutations. We have obtained these using hearts from mouse models, human cardiac tissue, and patient blood samples that are sources to create stem cells (human inducible Pluripotent Stem Cells, hiPSCs) that can be differentiated into patient-specific cardiomyocytes.

The partner Deliverable 2.1 that deals with data collected at the molecular and cellular level is presented at the same time with this Deliverable 2.2 that deals with the same information in terms of functional experiments on preparations with different level of complexity, from isolated myofibrils to the whole heart. The two partner tasks (2.1 and 2.2) have worked closely together throughout and the two deliverables should be read together. To emphasise the overlap and for clarity some material is reproduced in both reports. The data described here in Sections from 2.1 to 2.4 are functional data from cardiac preparations of mouse and, mostly, human models of HCM disease; Section 2.5 deals with the in vitro differentiation of cardiomyocytes from hiPSCs obtained from control subjects and FCM mutation carriers; Section 2.6 deals with the connection to WP5. The contents of the last two sections are reproduced both in D2.1 and D2.2.

Special focus has been given in WP2 to novel ideas about the contraction regulation mechanisms in striated muscle and the potential role of alterations of thick filament regulation and of the so called myosin Super Relaxed State (SRX) in the pathogenesis of HCM associated to mutations in myosin and MyBP-C [3-6]. For a more complete general Introduction and description of the role of the myosin SRX state, the reader is referred to the Introduction of D2.1.

1.2 Potential drugs targeting the molecular motor of the heart.

Functional data about the impact of three small molecules acting as potential drugs targeted at β-cardiac myosin SRX have been collected in the course of the project. Two of these drugs - Mavacamten (Mava), Omeocamtiv Mecarbil (OM) - have now completed stage 3 clinical trials and the UNIFI group was the EU coordinating center for one of the Mava trials [7,8]. In addition, the UW group has been the prime mover in the development of 2' deoxy-ATP (dATP) as a potential therapeutic agent [9-10]. We have published/submitted studies of two of these drugs as part of the SILICO funded work and presented a conference abstract about the third one [11-14] (see Section 2.3).

1.3 Alterations in Excitation-Contraction Coupling in HCM and additional therapeutic targets.

Though HCM is considered a disease of the sarcomere, because in most cases it is caused by mutations of one of the sarcomeric proteins, changes in cardiomyocyte electrophysiology and Ca^{2+} homeostasis have been reported to be part of all human HCM disease phenotypes [15]. These changes are not directly related to the sarcomeric mutations; rather they occur as adverse remodeling due to disease-associated alterations of cardiomyocyte signaling. Results from studies of the HCM alterations in cardiac electrophysiology and Excitation-Contraction Coupling in intact preparations from human and animal models of the disease are reported in Section 2.4. These studies are useful both for identifying additional therapeutic targets to treat the disease and for developing complete cardiac muscle models that are able to simulate the disease.

1.4 Induced Pluripotent Stem Cells from human patients with FCM.

To overcome the limitation of human heart samples to study HCM and test therapeutics, the UW (Seattle) group has led the development of human patient cardiac cell lines to be a platform for studying HCM associated mutations and testing small molecules and pharmaceuticals. UNIFI provided peripheral blood mononuclear cells (PBMCs) from 7 patients with a de novo mutation in cardiac MyBP-C (cMyBP-C) that results in HCM. PBMCs were shipped to UW where they were genetically reprogramed to become human inducible pluripotent stem cell lines that were subsequently differentiated into cardiomyocytes (hiPSC-CMs). Gene editing via CRISPR Cas9 has been performed on one patient hiPSC line to correct the mutation as an important isogenic control. Dr. Manuel Pioner (UNIFI) spent time in Seattle to learn and assist in this process. Several of the cell lines were shipped back to UNIFI and they, along with the UW group, are culturing the hiPSC-CMs to perform contractile performance and electrophysiological experiments on cells and sub-cellular contractile organelles (myofibrils). These experiments were delayed by several months due to the COVID-19 pandemic, but currently multiple batches of hiPSC-CMs are being cultured out to 30-90 days for experiments (see Section 2.5). This work is reported in Section 2.5 where parallel studies have begun on patient myectomy samples at UNIFI (within Task 2.2) and the same patient's derived iPSCs in UW (within Task 2.1).

This section is reproduced in both deliverable reports (D2.1 & D2.2) to emphasise the success of this cross Task collaboration.

1.5 Collaboration with WP5 MUSICO.

It is essential that the data generated in WP2 (Task 2.1 and 2.2) feeds directly and continually into the development of modelling software packages in WP5 (Figure 1).

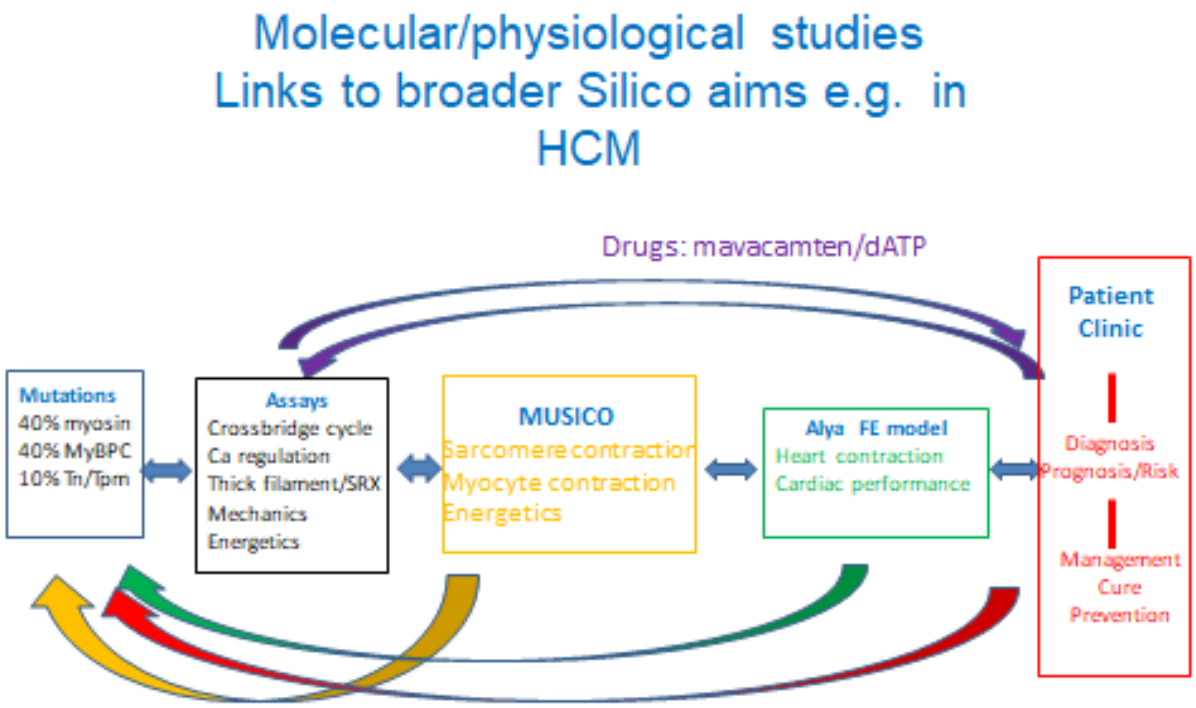


Figure 1. Relationship of WP2 (blue /black boxes) with the broader SILICOFCM project and the modelling packages of WP5 (yellow/green boxes).

The double headed arrows indicate the two-way exchange of information between each level of the project from the molecular studies of the mutations on the left to the patient studies on the right. At each stage there is feedback from the modelling to drive the collection of new experimental data to provide testing and validation of the modelling. Similarly, there is feedback between the molecular and functional studies and the patient data emphasised by parallel studies of therapeutic molecules exemplified here by small molecules acting on the myosin motor.

The MUSICO package included units to model (i) the crossbridge cycle and its regulation in solution, (ii) contraction of a single sarcomere, (iii) whole cardiomyocyte and cardiac tissue contraction which further feeds into whole heart models. The work of WP2 has been tightly coordinated with WP5 and has made extensive use of the MUSICO simulation package to analyze data (see Section 2.6) and data from the WP2 members have been used to develop MUSICO packages for the modelling of cardiac twitch contractions in normal myocytes/trabeculae and in those carrying mutations in the sarcomeric proteins (see also Section 2.1). Section 2.6 describes the jointly published work of WP5 and WP2 that includes major contributions within both Task 2.1, Protein & Cell Data and Task 2.2, Physiology Data. This part of the report appears in both D2.1 and D2.2.

1.6 Published outputs.

The experimental science completed under SILICOFCM in WP2, Task 2.2 is summarized in Section 2 below and, in most cases, it has been published or submitted as full papers (see Appendix 1); in some cases, it has been submitted as conference presentations and the corresponding papers are in preparation (Appendix 1). In a few cases the work is incomplete and will continue beyond the submission of this deliverable and beyond the end of the SILICOFCM project. Some of the still unpublished studies are described in Section 2 whereas the future plans are outlined in Section 5.

2. Results from Physiological Experiments

Disease mechanisms responsible for genetic cardiomyopathies, with particular focus on HCM, have been investigated in experimental preparations of different complexity from human and animal models.

The impact of specific mutations, most often associated to HCM, on sarcomere mechanics kinetics and energetics has been identified.

Secondary, adverse, remodeling of cardiomyocyte electrophysiology and Excitation-Contraction Coupling has been investigated.

Both research lines have led to identifying potential therapeutic targets and testing the effects of specific drugs.

A novel *in vitro* model system to investigate the impact of genetic cardiomyopathies at early stages on both sarcomere and excitation-contraction coupling functions based on human inducible pluripotent stem cells from patients has been developed and started to be tested for one specific HCM mutation.

Most results from functional experiments will be used to test and improve the MUSICO platform.

2.1 Studies of transgenic mice with HCM- and DCM-like mutations.

Troponin is the protein complex in the sarcomeres that is the gate keeper for initiation and regulation of heart muscle contraction during systole. Mutations in troponin that increase or decrease its responsiveness to rises in intracellular calcium (that triggers contraction) are responsible for hypertrophic (HCM) and dilated (DCM) cardiomyopathy, respectively. Physiological measures in transgenic mice expressing an HCM-like (L48Q) and DCM-like (I61Q) cardiac troponin C (cTnC) mutations have increased and decreased response to calcium and demonstrate hyper- and hypo-ventricular systolic function and have ventricular structure remodelling consistent with these diseases [16]. The UW group has also recently reported that transgenic over-expression of L48Q cTnC can rescue the dilated cardiomyopathy phenotype of a mouse with a different DCM related mutation, e.g. tropomyosin D230N [17]. Since these mutations have not been identified in patients, it suggests that the altered calcium responsiveness can drive the disease initiation and progression.

In mice and in humans with these diseases, the amount of mutation incorporated into muscle sarcomeres is variable and $\leq$ 50% of the total TnC, which can make interpretation of experimental findings and treatment regimens challenging. To overcome this, we have used MUSICO to study how variable mutant incorporation into sarcomeres alters the contractile properties of heart muscle. Experimentally we measured contractile properties of heart tissue from these transgenic mice to determine the effect of partial incorporation of each mutation on muscle and cardiac function. For L48Q TnC mice mutant incorporation was 30% and for I61Q cTnC mice incorporation was approximately 50%. Measurements included echocardiography, intact cardiac muscle twitches, and demembranated muscle calcium dependence of force generation.

These data were used in WP5 to determine the parameter adjustments that allowed good simulation of the data [18] (see also Section 2.6.2). Adjusting parameters associated with altered calcium binding provided excellent fits to the experimental data from both rat (Figure 2) [19] and mouse (Figure 3) cardiac trabeculae, thus providing validation for MUSICO's multi-scale models.

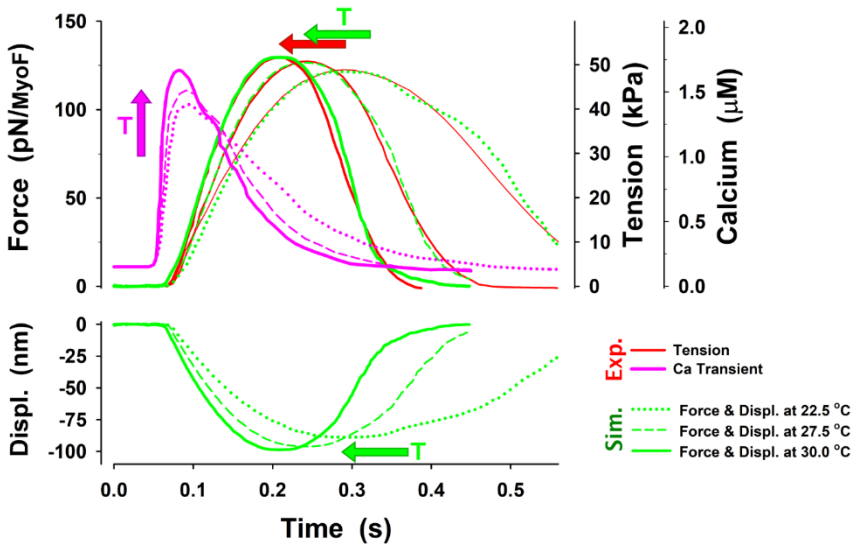


Figure 2. Effect of temperature on Ca^{2+} transient and twitch force in rat ventricular trabecula under fixed-end isometric conditions: experimental tracings versus MUSICO model predictions.

The MUSICO simulation with the six state model (green lines) matched well the force responses (red lines) at 22.5 °C, 27.5 °C and 30 °C [20]. The rise of temperature increased peak calcium concentrations and caused a faster rise and fall of the transient (pink lines). The force transients followed similar patterns having faster rise times, modest increase in peak force and a faster relaxation phase. The good match to the experimental observations required increase in binding and ADP release rates with increasing temperature.

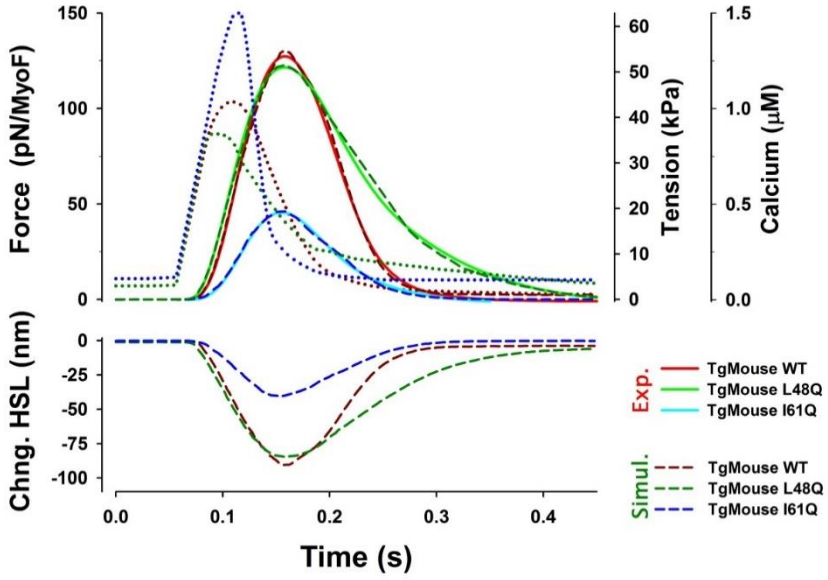


Figure 3. Twitch contractions for WT, I61Q and L48Q trabeculae from transgenic mice (TgMouse) compared to MUSICO predictions.

Model predictions of twitches (dashed lines) at initial sarcomere length 2.3 µm are superimposed on the experimental twitches (solid lines) for WT (red), L48Q (green) and I61Q (blue). Best fit model parameters are reported in [18]. The twitches are driven by the Ca2+ transients (dotted lines) [16], and model estimates of half sarcomere length changes (Chng. HSL).

MUSICO was then used to study the effect of different levels of mutant incorporation into mouse cardiac sarcomeres as well as the effect of different patterns of incorporation within and between sarcomere thin filaments (Figure 4 and 5).

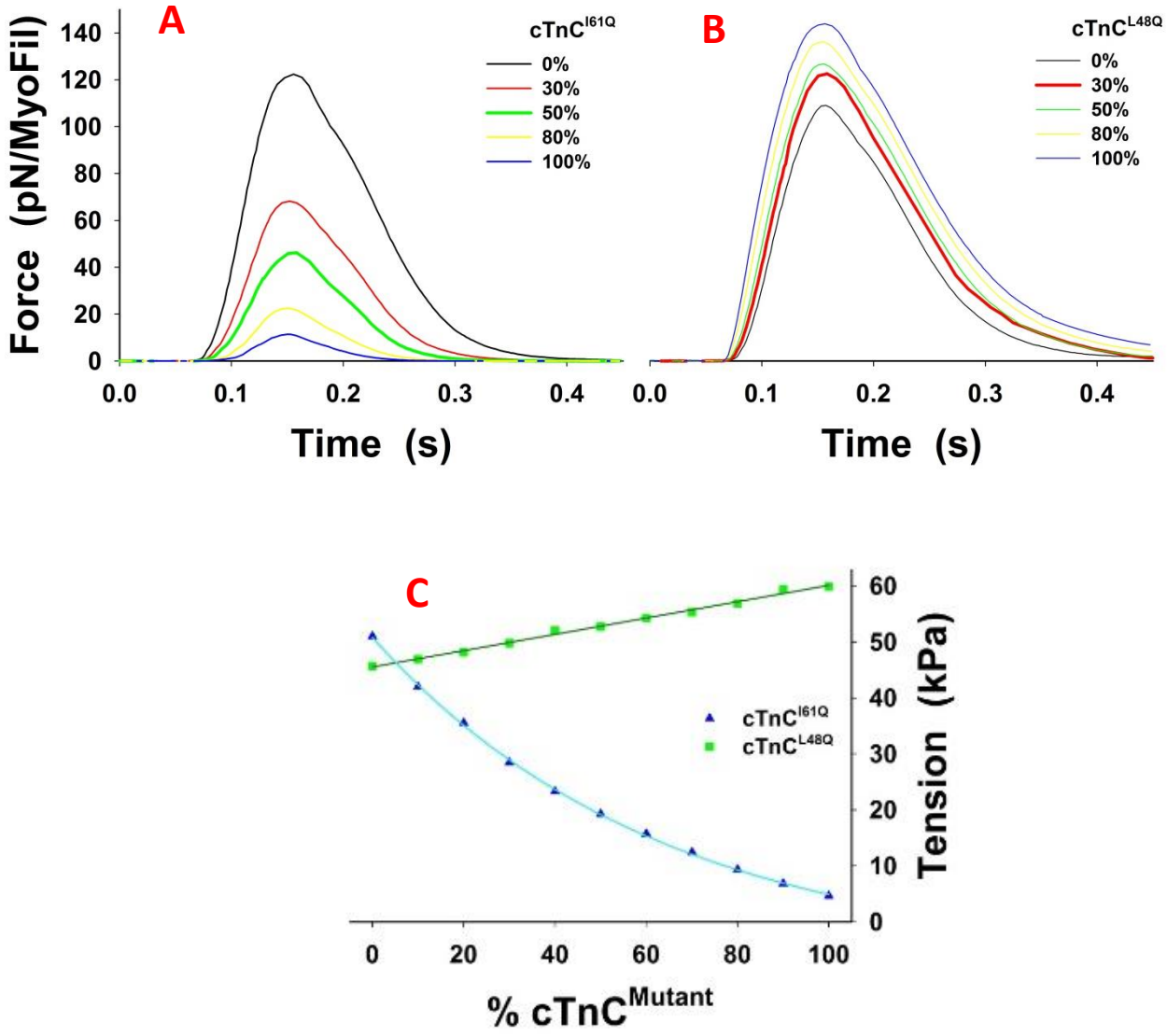


Figure 4. Model predicted impact of mutant protein penetrance on peak twitch tension.

Figure 4 shows predicted effect of % incorporation of $cTnC^{I61Q}$ (A) and $cTnC^{L48Q}$ (B) on twitches as well as (C) peak tension as a function of % incorporation (Δ) $cTnC^{I61Q}$, (□) $cTnC^{L48Q}$.

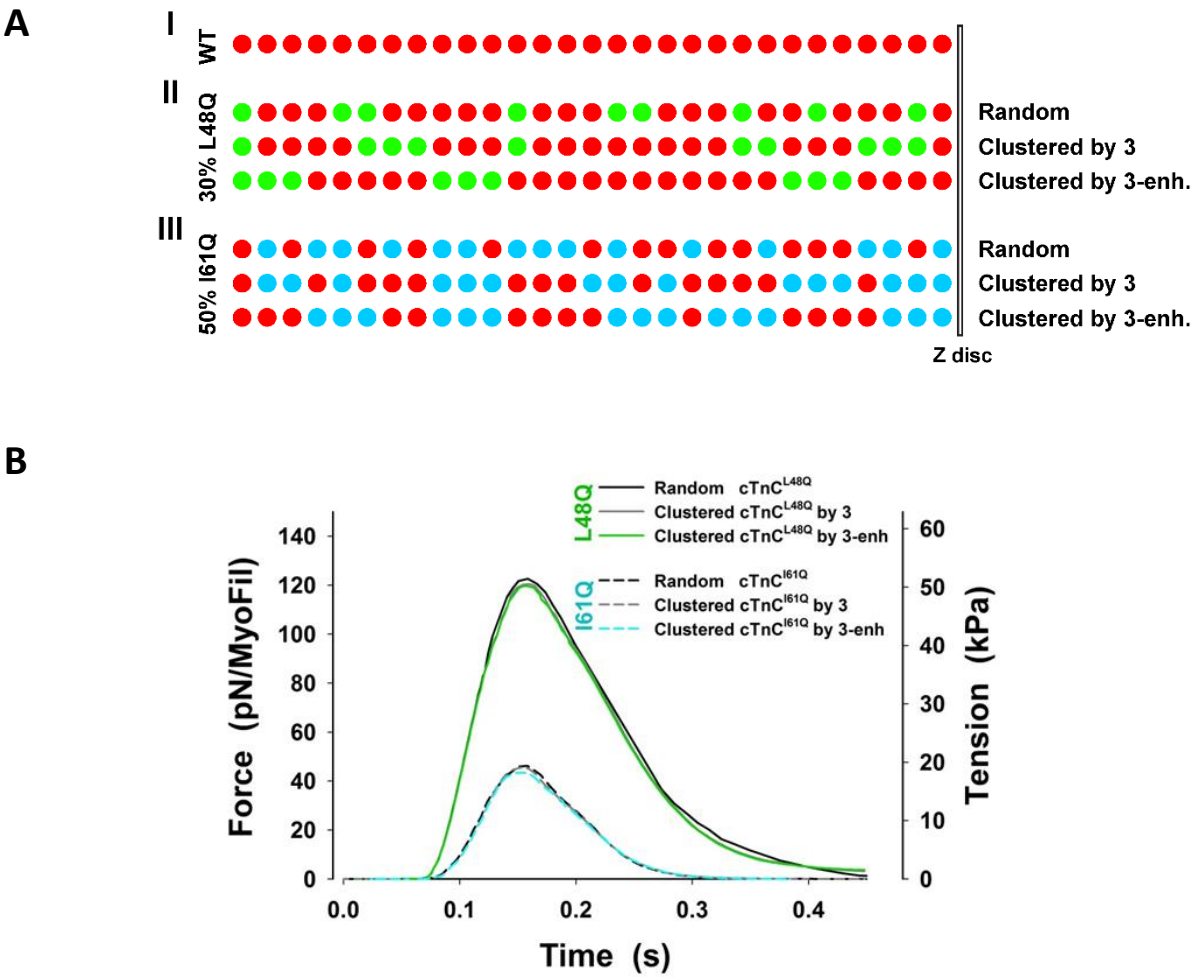


Figure 5. The effect of clustering of mutated TnC's in each actin filament.

Part A of Figure 5 shows different random distributions of mutant TnC within a representative strand of an actin filaments if mutant TnCs tend to cluster: I. cTnCWT, II. random and clustered distributions of 30% cTnCL48Q and III. random and clustered distributions of 50% cTnCI61Q. In II and III the distribution includes a significant number of clusters of three mutated cTnCs, denoted as "Clusters by 3" and further enhanced number of the clusters of three, by ~ 25%, denoted as "Clusters by 3-enh." Part B of Figure 5 shows the effect of mutant cTnC distributions on tension transients where the random distribution simulation data (from Figure 4) is compared with clustering cTnCL48Q (solid lines) and cTnCI61Q (dotted lines) by 3 or by 3-enh, keeping partitioning as shown in (A).

These variations are likely in patients with HCM or DCM but are quite challenging to study experimentally, thus demonstrating the versatility and value of the MUSICO platform. These computational simulations, driven by the experimental data are reported in a manuscript that is currently in review [18].

2.2 Data collection from myofibrils isolated from human HCM samples about the impact of specific mutations on sarcomere function.

Mechanics and energetics of human cardiac sarcomeres from a homozygous HCM patient carrying a mutation in cTnT (K280N) have been investigated and compared with those of different groups of control patients [21]. Force generation and apparent cross bridge kinetics were investigated in isolated myofibrils whereas sarcomere ATPase was simultaneously measured with isometric tension in skinned multicellular cardiac strips (Figure 6).

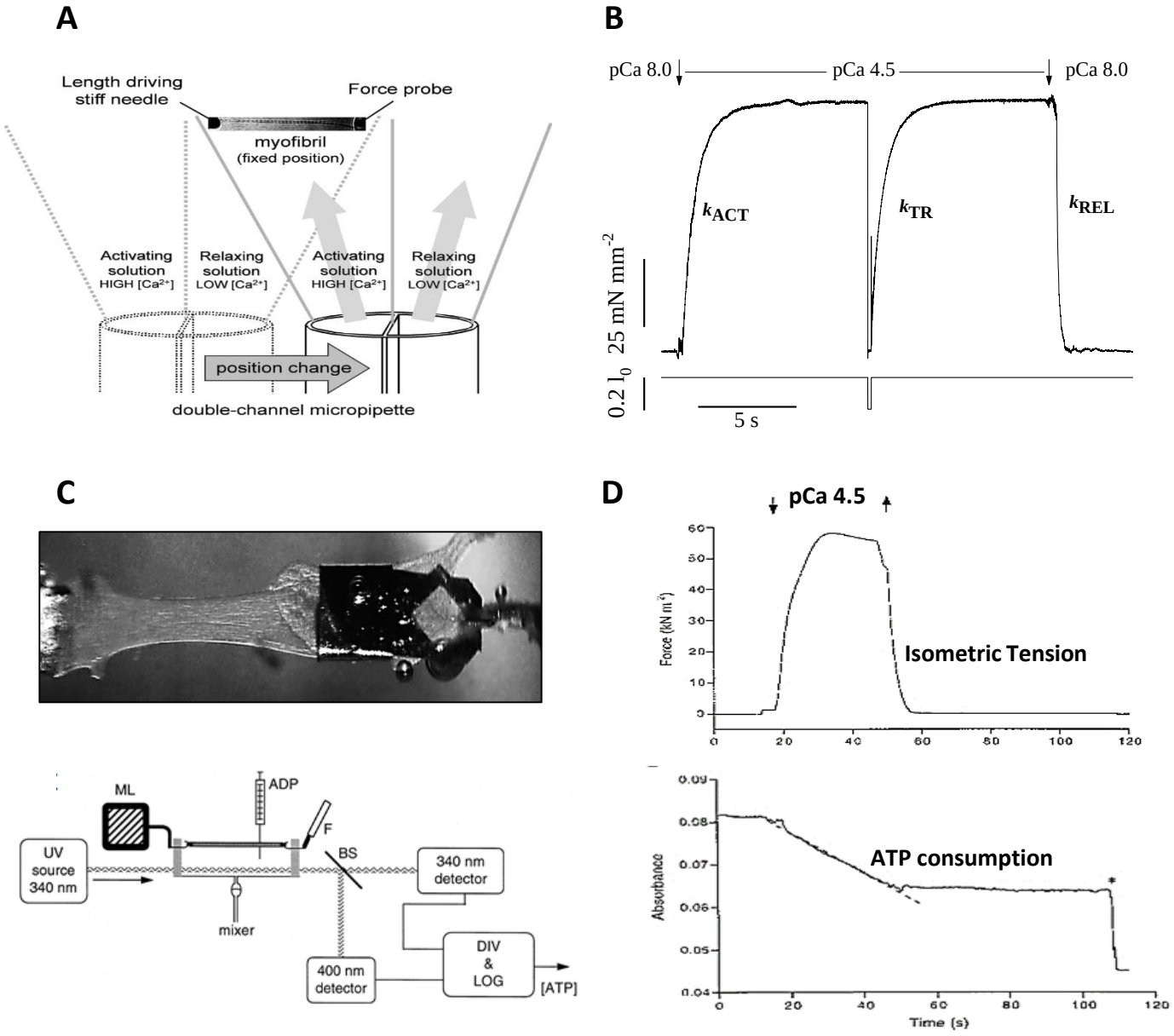


Figure 6. Methods to measure human cardiac myofibril mechanics & kinetics and human cardiac trabecula mechanics & energetics from patient samples.

Part A shows isolated myofibrils mounted between a cantilever force transducer and a length control motor are maximally calcium-activated and fully relaxed by fast solution switching between high calcium (pCa 4.5) and nominally calcium free (pCa 8.0) solutions. Part B shows what happens following sudden calcium activation, the myofibril develops force with a rate - kACT - that has been shown to reflect apparent crossbridge turn-over. Under steady-state conditions of activation, a fast release-restretch perturbation is applied to the myofibril to detach force generating crossbridges and start a force redevelopment process with rate kTR. Following calcium removal, the myofibril force decays to zero with a biphasic time course that allows us to measure an early slow and a later fast kREL rates (e.g. [22]) Parts C & D shows simultaneous measurements of isometric force and ATP consumption can be performed in multicellular permeabilized cardiac preparations from human ventricular samples using previously published technique (e.g. [23]). Briefly, ATPase activity was measured using an enzyme coupled assay in which ATP regeneration from ADP and phosphoenol-pyruvate by the enzyme pyruvate kinase is coupled to the oxidation of NADH to NAD and the reduction of pyruvate to lactate by L-lactic dehydrogenase. NADH oxidation was photometrically measured from the absorbance at 340 nm of near-UV light. The maximal Ca2+-activated ATPase activity was calculated by normalizing the maximal NADH oxidation to the volume of the muscle strip and subtraction of the basal NADH oxidation (basal ATPase activity) normalized to muscle strip volume. Basal NADH oxidation was measured in relaxing solution.

In one group of experiments the mutant protein has been almost completely replaced with a control wild type troponin to check the direct impact of the mutant protein on sarcomere function. Results from myofibrils of the HCM homozygous patient and different controls and results of Tn replacement experiments are reported in Table 1. The results show that the mutation accelerates cross bridge kinetics, especially the apparent isometric rate of cross bridge detachment, impairing cardiomyocyte energetics by increasing the energetic cost of isometric tension generation. In addition, the mutation may also increase the energetic cost of diastole, likely, by altering the mechanisms that switch the thin filaments off following Ca^{2+} removal and, therefore, causing diastolic dysfunction.

Table 1. Mechanical and kinetic parameters of myofibrils from the homozygous K280N cTnT HCM patient and different groups of control patients (top 4 rows of data) **and impact of cTn exchange in K280N and donor myofibrils** (bottom 6 rows of data)**.**

Myofibril type	**RT**	**Po**	**k_{ACT}**	**D_{slow}**	**slow k_{REL}**	**fast k_{REL}**
	kN m^{-2}	**kN m^{-2}**	**s^{-1}**	**ms**	**s^{-1}**	**s^{-1}**
Donors (N=5)	10.4±0.6 (n=98)	111±5 (n=96)	0.85±0.02 (n=118)	186±5 (n=100)	0.29±0.02 (n=95)	4.18±0.14 (n=101)
LVH_{ao} (N=7)	9.8±1.1 (n=48)	96±6 (n=58)	0.71±0.04 (n=50)	248±13 (n=54)	0.20±0.02 (n=54)	3.15±0.20 (n=54)
HCM_{smn} (N=3)	8.2±0.9 (n=44)	87±7 (n=43)	0.74±0.03 (n=45)	206±7 (n=44)	0.30±0.02 (n=44)	4.62±0.20 (n=44)
K280N (N=1)	**14.9±1.4*** (n=43)	87±5 (n=43)	**1.73±0.07*** (n=39)	226±11 (n=43)	**0.63±0.05*** (n=42)	3.76±0.16 (n=42)
Tn exchanged batches						
K280N sham treated (N=1)	15.9±2.9 (n=12)	89±9 (n=12)	1.69±0.08 (n=17)	240±16 (n=16)	0.69±0.08 (n=16)	3.90±0.27 (n=16)
K280N WT Tn exch (N=1)	15.7±2.0 (n=13)	86±7 (n=14)	**1.00±0.09^{++}** (n=16)	259±27 (n=15)	**0.31±0.05^{++}** (n=16)	**3.13±0.19^{+}** (n=16)

Donors sham treated (N=2)	10.5±0.83 (n=64)	114±7 (n=62)	0.86±0.03 (n=70)	173±6 (n=62)	0.28±0.02 (n=58)	4.26±0.18 (n=63)
Donors WT Tn exch (N=2)	10.1±0.7 (n=72)	**91±6**$^{+}$ (n=63)	0.84±0.03 (n=70)	179±8 (n=59)	0.30±0.02 (n=66)	4.65±0.19 (n=65)
Donor sham treated (N=1)	10.8±1.1 (n=14)	94±10 (n=14)	0.81±0.04 (n=15)	214±7 (n=15)	0.33±0.04 (n=15)	3.78±0.32 (n=15)
Donor K280N Tn exch (N=1)	**15.9±1.9**$^{+}$ (n=12)	77±9 (n=10)	**1.04±0.08**$^{++}$ (n=12)	**186±9**$^{+}$ (n=12)	**0.60±0.07**$^{++}$ (n=13)	4.18±0.62 (n=13)

Error! Reference source not found.1 shows means ± SE, N, number of patients in the group; n, number of myofibrils; RT, resting tension; P_0, maximal Ca^{2+}-activated tension; k_{ACT}, rate of force generation following maximal Ca^{2+}-activation estimated from the time constant of the exponential fit to the force trace; D_{slow}, duration of the slow isometric phase of relaxation following sudden Ca^{2+} removal; slow k_{REL}, rate of the slow isometric phase of relaxation estimated from the normalized slope of the linear fit to the force trace; fast k_{REL}, rate of the fast phase of relaxation estimated from the time constant of the exponential fit to the force trace. For the statistics of the comparison between groups in the first 4 rows see Supplemental material in [21]; * K280N parameters for which the statistical analysis shows a significant difference vs all control groups. For the statistics of the cTn exchange experiments (bottom 6 rows): + P<0.05 ++ P<0.01 Student's t test for unpaired observations (exchanged myofibrils vs sham treated unexchanged myofibrils).

Mechanical and energetic experiments were also performed on human cardiac samples from a few HCM patients carrying the E258K missense mutation in the *MYBPC3* gene [24,25]. The cMyBPC variant is a founder mutation in Tuscany (Figure 7) and blood cells from these patients have been used to start the experiments described in section 2.5.

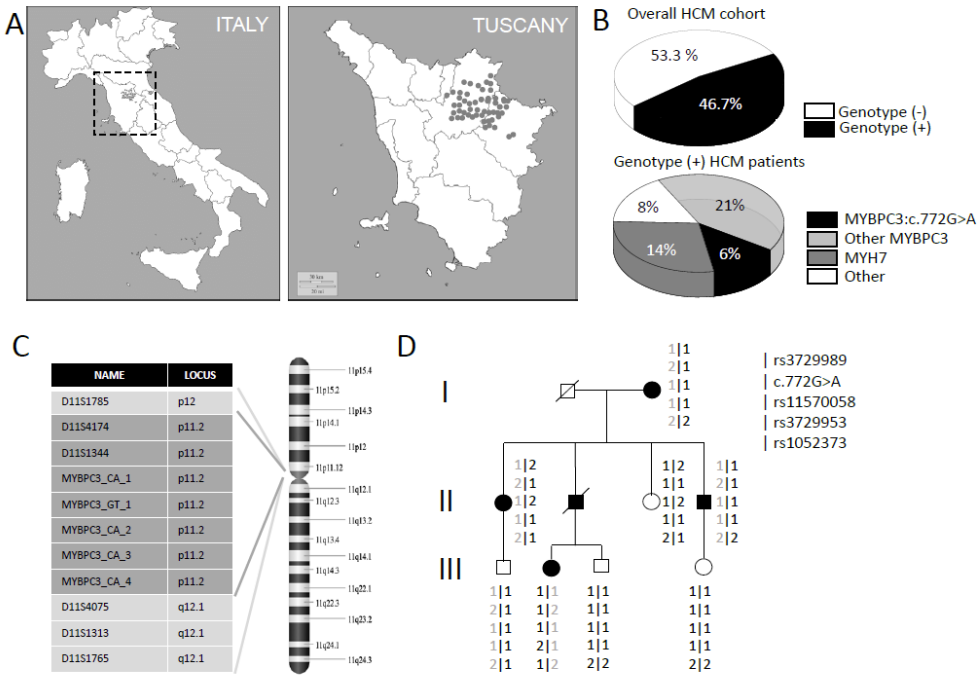


Figure 7. Prevalence of the c.772G>A (E258K) mutation in MYBPC3 in the Florence HCM cohort and the demonstration of a founder effect.

Part A shows geographical origin of HCM patients carrying the c.772G>A (E258K) variant who underwent genetic testing. Part B shows prevalence of genotype positive (obtained considering sarcomeric variants in MYBPB3, MYH7, TNNT2, TNNI3, MYL2, MYL3, TPM1 or ACTC1 classified as pathogenic/likely pathogenic/of uncertain significance as per guidelines available at the time of testing; [26]) and genotype negative patients among the overall HCM Florence Cohort (top). Prevalence and distribution of sarcomeric myofilament protein gene mutations among the 46.7% genotype-positive HCM patients. Parts C and D show PCR assays for five intragenic microsatellite markers (CA/GT dinucleotide repeats were designed, and their products sequenced to obtain genotypes for segregation analysis. Numbers were assigned randomly to repeat alleles, and haplotypes were reconstructed with Merlin. The “grey" haplotype marked is shown to segregate consistently with HCM in an illustrative large pedigree.

While the myocardium from the cTnT mutant patient described above, expressed 100% of the mutant protein, cardiac samples from the E258K cMYBPC patients expressed only the WT protein but at levels significantly lower than those of controls (haplo-insufficiency; see also section 2.5). The results - obtained from mechanical and energetic measurements in myofibrils and skinned trabeculae- showed that decreased expression of cMyBPC affects sarcomeric function by increasing the apparent rate of cross bridge detachment under isometric conditions and the energy cost of isometric tension generation (Figure 8 and 9) as reported for the cTnT mutation described above [21] and previously reported for the R403Q myosin mutation [22,23].

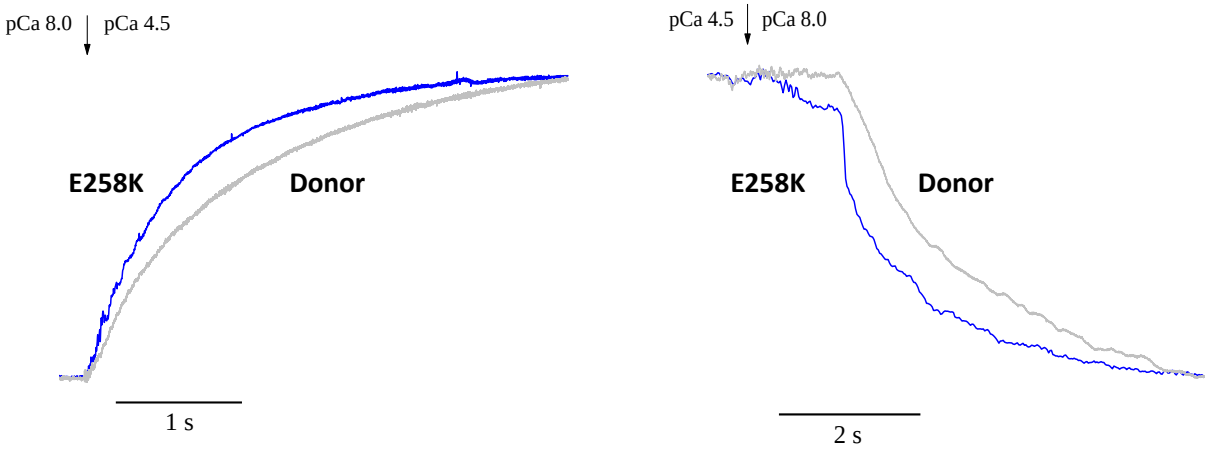


Figure 8. Impact of E258K *MYBPC3* mutation on myofibril mechanics and kinetics.

Representative force responses of donor and E258K myofibrils maximally activated (pCa4.5) and fully relaxed (pCa8.0) by fast solution switching technique. The tension curves from E258K and donor myofibrils are normalized and superimposed to highlight the kinetics of force generation and relaxation

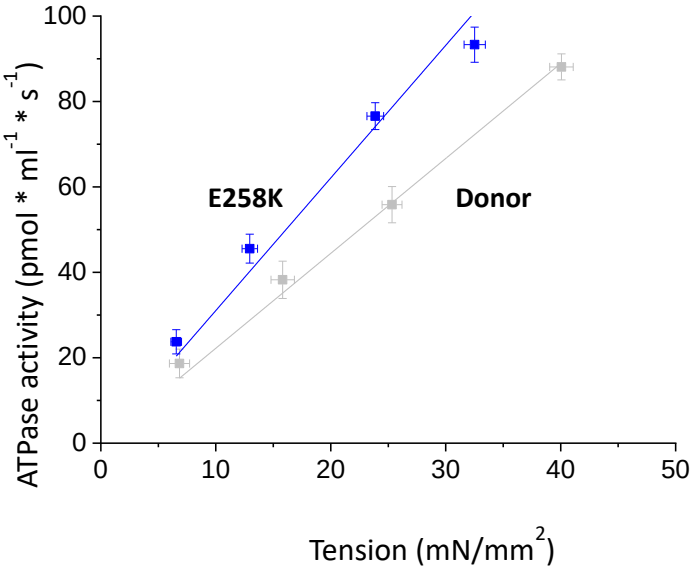


Figure 9. Impact of the E258K mutation on sarcomere energetics.

Average pooled rate of ATP consumption vs force relations (tension cost) in E258K and donor trabeculae (E258K N=3, n=13; donor N=2, n=13) (y = 3.04x and y = 2.08x respectively). Data were pooled in 10% wide steady state force bins and were fit by linear regression as indicated by the solid line

In the present study a technique is also described that, following a tissue clearing protocol, allows a reconstruction of the 3-D structure of human cardiac trabeculae that may be of help in checking HCM-related disarray of cardiomyocyte bundles.

As reviewed in [27], the results obtained from a number of cardiac samples of HCM patients expressing mutations in different sarcomeric proteins supports the hypothesis that excess/inefficient ATP utilization by myofilaments plays a central role in the pathogenesis of HCM. This disease mechanism must be taken into account by both therapeutic strategies and cardiac modelling work.

2.3 Effects of small molecules acting on myosin motor function that are good candidates for cardiomyopathy therapy.

To date, pharmacological treatment options of genetic cardiomyopathies are not disease-specific and rather focus on managing the symptoms, without addressing the disease mechanisms. Earliest attempts at improving cardiac contractility by modulating the sarcomere indirectly (inotropic drugs that affect cardiomyocyte Ca^{2+} cycling) in DCM and Heart Failure resulted in unwanted effects. In contrast, targeting the sarcomere directly, in particular the myosin heavy chain (the molecular motor of the heart), led to identifying small molecules with some therapeutic value in cardiac muscle disorders. In this workpackage, the impact of some of these molecules has been tested in *vitro* in the cardiac muscle of human and animal models.

The MUSICO platform will be used to simulate and predict the effects of some of the small molecules at multiple scales, from protein to whole ventricle. These simulations will be a validation tool for the

models at multiple scales and will contribute to the development of a model platform that can test pharmaceutical agents as potential therapeutics.

In addition to simulations, some of the small molecules will be also tested in iPSC-cardiomyocyte cell lines from genetic cardiomyopathy patients (see section 2.5)

2.3.1 Mavacamten, a small molecule that may improve cardiac function in HCM.

Mavacamten (MYK-461) is a promising small molecule designed to act as an allosteric inhibitor of sarcomeric myosins [28] and has already been used in clinical trials for treatment of HCM [6]. We have characterized Mavacamten effects in human ventricular myofibrils compared to fast skeletal myofibrils from rabbit psoas. The results are reported in a manuscript under revision at the J Gen Physiol [13] and had been presented in abstract form in a few meetings [29]

Mavacamten has a fast, fully reversible and dose dependent negative effect on maximal Ca^{2+} -activated isometric force in both myofibril types with a sensitivity that is one order of magnitude higher in human cardiac myofibrils (Figure 10). In fast skeletal myofibrils Mavacamten was also found to decrease the kinetics of force development while in human ventricular myofibrils, Mavacamten had no negative effect on the rate of force generation. Mavacamten in fast skeletal muscle did not alter force relaxation while it had a strong positive lusitropic effect on human ventricular myofibrils that suggested a positive impact on diastolic function.

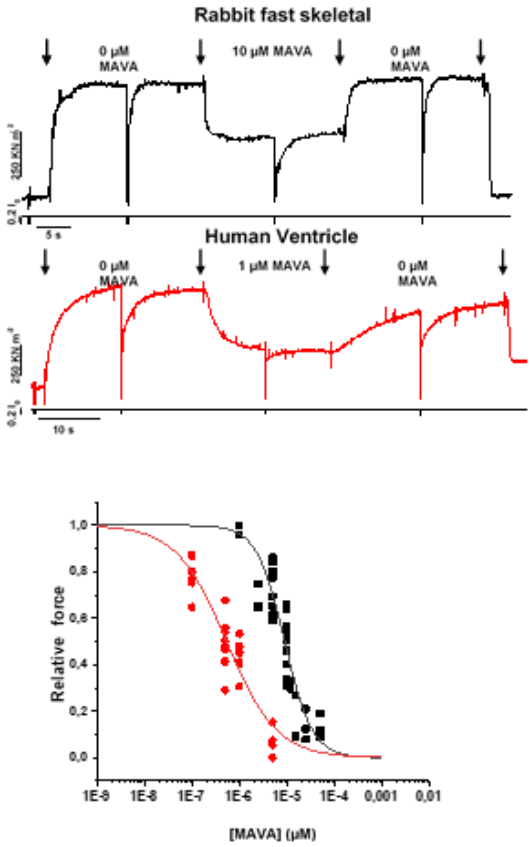


Figure 10. Mavacamten (MAVA) decreases maximal Ca^{2+} activated force of skeletal and cardiac myofibrils.

Representative MAVA-jump traces from fast skeletal (black) and human ventricular (red) myofibrils. Arrows mark the fast solution changes as indicated. Bottom: MAVA dose-response relationships for maximal Ca2+-activated tension (pCa 4.50) obtained during the "jump" protocols in skeletal (black) and cardiac (red) myofibrils. Cardiac myofibrils are one order of magnitude more sensitive to MAVA compared to fast skeletal myofibrils.

In human cardiac myofibrils, the impact of the time of exposure to the drug on the kinetics of force recovery following sudden drug removal provides functional hints that Mavacamten action may be mediated by the shift of detached motor heads towards auto-inhibited SRX state. The ensemble of these effects outlines a motor isoform-specific dependence of the inhibitory effect of Mavacamten on force generation that may be of great value in the treatment of HCM.

The impact of Mavacamten on the energetics and excitation contraction coupling of human cardiac muscle and cardiomyocytes is currently under investigation.

Results from these experiments will be simulated by the MUSICO platform also to contribute providing a better model description of the crossbridge transitions between Disordered and SRX states.

2.3.2 Omecamtiv, a small molecule that may improve cardiac function in DCM.

Omecamtiv mecarbil (OM) is a small molecule proved to enhance the function of the beta-myosin motor [30] and presently used in patients for the treatment of heart failure [8]. Despite the positive outcomes of clinical trials, the mechanistic basis of its action are still controversial and very little is known of its functional impact in human cardiac sarcomeres.

In WP2 the UNIFI group has used myofibrils from frozen samples of human donor ventricles to study the effects of µM OM on isometric force in relaxing conditions (pCa 9) and at maximal (pCa 4.5) or half-maximal (pCa 5.75) calcium activation, under control experimental conditions (15°C, equimolar DMSO, contaminant [Pi] ~ 170µM) or in the presence of 5 mM Pi. In all conditions tested, OM increased isometric force with a complex dose-dependent effect that peaked at 0.5 µM (about 40% force increase) and never decreased below control values (Figure 11). As already reported in human cardiac muscle [31], OM strongly depressed the kinetics of force development up to 90% at a drug concentration of 10 µM (Figure 11). Of note, OM was found to decrease the inhibition of force by Pi, in agreement with observations in rabbit soleus muscle [32], but only slightly and at low [Pi] (1-2 mM). In relaxing control conditions, OM induced a calcium-independent force development which approached the maximum calcium activated tension level at 10 µM OM. In the absence of calcium, the kinetics of force development was very slow and independent of the drug concentration, tending to the rates measured in the presence of activating calcium and high [OM]. The results obtained in human cardiac myofibrils agree with OM involvement in a complex perturbation of the thin/thick filament regulatory states of the sarcomere. OM mediates strong myosin motor binding via an allosteric mechanism coupled to Pi release.

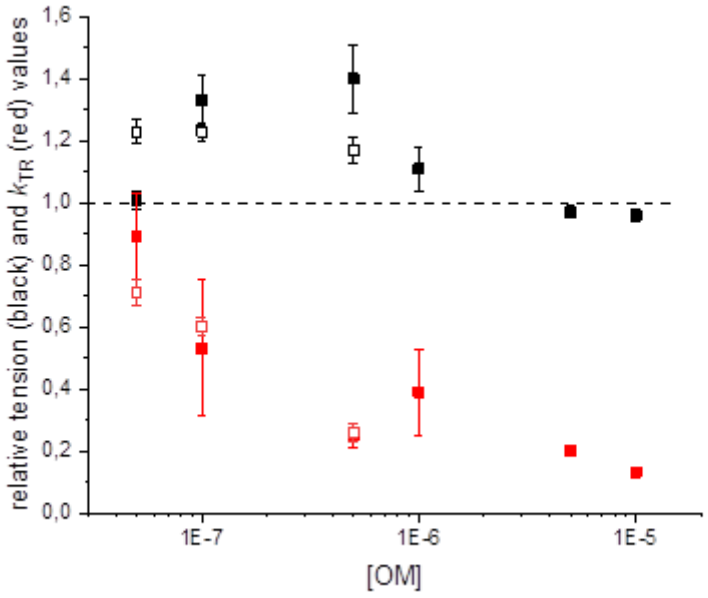


Figure 11. Dose dependent effects of Omeocamtic Mecarbil (OM) on Ca^{2+}-activated tension and rate of tension redevelopment (k_{TR}) of human donor ventricular myofibrils.

Tension (black symbols) and k_{TR} (red symbols) values are normalized to those measured in the absence of the drug. Filled symbols are data taken from maximal Ca^{2+}-activated contraction (pCa 4.50); empty symbols are data taken from submaximal Ca^{2+}-activated contraction (pCa 5.75 i.e. close to pCa_{50}). k_{TR} has been measured under steady state conditions of Ca^{2+}-activation following a fast release-restretch maneuvre.

The results have been submitted in abstract form at the 2021 Biophysical Society Annual Meeting [14]. A full paper reporting these results is in preparation.

The impact of OM on skinned and intact human cardiac trabeculae and intact cardiomyocytes is currently under investigation.

2.3.3 2’ deoxy-ATP (dATP), a small molecule that enhances cardiac function in heart failure.

The UW group has previously demonstrated that elevating the level of dATP (a naturally occurring nucleotide) in failing myocardium, resulting from myocardial infarct, rescues cardiac function in rodent [10] and pig [9] models. Other previous studies (reviewed in [33,34]) provide a firm mechanistic basis, demonstrating that when cardiac myosin uses dATP in place of ATP there is an increase in myosin activation and faster actin-myosin (crossbridge) cycling. In a just published report [11] we demonstrated how dATP can keep muscle primed for rapid myosin activation during the period of relaxation between muscle contractions. The UW group and collaborators have simulated the effect of dATP on myosin structure [26, 35] and how dATP effects on myosin-actin ‘crossbridge’ cycling drives increased twitch contractions in heart muscle [12].

These were precursors for modelling with the MUSICO platform. We are currently simulating the effects of dATP at multiple scales, from protein to whole ventricle. Since we have experimental data at all scales as a comparator, these simulations will act as another validation tool for modelling at each

scale and across scales. They also fulfil the main goal of SILICOFCM – to develop a computational modeling platform that can test small molecules and pharmaceutical agents as potential therapeutics.

Finally, we will test dATP in the newly developed patient iPSC-cardiomyocyte cell models of HCM (E258K-MYBPC3 mutation) created for the SILICOFCM project (see section 2.5). Cardiomyocytes will be transduced with viral vectors that result in over-expression of the enzyme Ribonucleotide Reductase, which converts ATP to dATP. We will determine how this affects the contractile properties of the patient cardiomyocytes, to determine efficacy in a human disease model.

2.4 Data collection from intact cardiac preparations from human HCM samples about the impact of the disease on Excitation-Contraction Coupling and the use of negative inotropic drugs.

The UNIFI group previously analyzed the electromechanical profile of cardiomyocytes isolated from myectomy samples of obstructive HCM patients [15, 36]. When compared with control cells, HCM cardiomyocytes showed prolonged action potentials, frequent afterdepolarizations, slower Ca^{2+}-transients and elevated diastolic Ca^{2+} concentration, largely determined by overexpression of the late Na^{+} current (I_{NaL}). Indeed, these electro-mechanical abnormalities were reversed by the I_{NaL} inhibitor ranolazine, with beneficial effects on diastolic function and cellular arrhythmias.

Disopyramide (Diso) is a potent negative inotrope. It has been used to decrease left ventricular outflow tract obstruction in obstructive HCM since first reports in the early 1980's. Despite its active use since the 1980's, there has been little work on the intracellular mechanism of therapeutic effects of Diso beyond its categorization as a type I antiarrhythmic, that is, as a sodium channel blocker. Considering its potent negative inotropic effects, it is not known whether the drug has additional direct effects on Excitation-Contraction Coupling or on the actin-myosin interaction and the effects of Diso in human HCM cardiomyocytes have not been characterized. We found that Diso exerts a wide range of channel blocking activity in human HCM myocardium, accounting for its negative inotropic effect, with no direct myofilament inhibition. Its complex electrophysiological profile does not exert proarrhythmic effects *in vitro*. Rather, it shows potential for reduced ventricular arrhythmic propensity. Diso is an effective negative inotrope while at the same time avoiding interference with sarcomere protein function. As such, it does not appear incompatible with the new class of allosteric myosin modulators (e.g. Mavacamten) currently under investigation. This promises to enrich the pharmacological armamentarium to control obstruction as well as arrhythmias in HCM. The results of this study have been published in [37] (Figure 12).

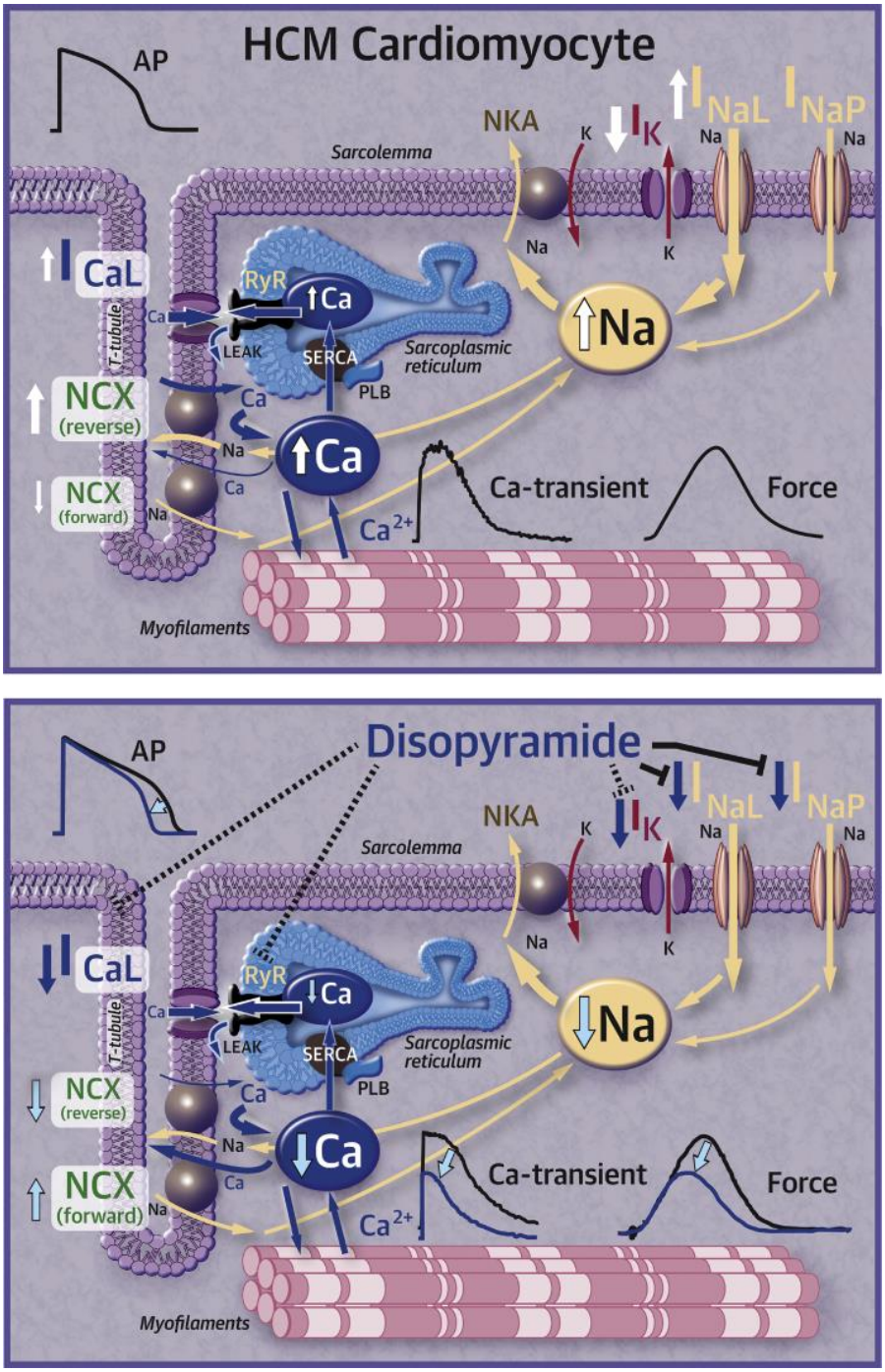


Figure 12. Effects of Disopyramide in HCM Cardiomyocytes.

In HCM cardiomyocytes, $I_{Ca\text{-}L}$ and I_{NaL} are increased, while IK is markedly decreased, leading to prolonged APs; Na overload impairs NCX, contributing to cytosolic Ca-overload. Disopyramide inhibits $I_{Na\text{-}peak}$ (I_{NaP}), I_{NaL}, $I_{Ca\text{-}L}$ and I_K, while also stabilizing ryanodine receptors. These effects lead to shortening of APs. Moreover, normalization of NCX function and $I_{Ca\text{-}L}$ inhibition and RyR stabilization contribute to reduce diastolic Ca and systolic Ca-release, determining negative inotropic effects. APs = action potentials; $I_{Ca\text{-}L}$ = L-type Ca current; I_K= delayed-rectifier K current; I_{NaL} = Late Na current; NCX = Na+/Ca^{2+} exchanger; RyR = ryanodine receptor.

We previously reported a characteristic and disease-specific E-C coupling remodelling in human and mouse models of HCM, with marked alterations of T-tubule architecture [15, 38]. In this project we

reviewed the features of T-tubule remodelling in HCM and their implications in terms of mechanical dysfunction and arrhythmogenesis [39]. Evidence is given to support the idea that pathological T-tubule remodelling in HCM hearts is disease-specific and may also present some mutation-specific features. A comparison with data in acutely de-tubulated myocardium is presented to highlight how a shift from synchronous Sarcoplasmic Reticulum Ca^{2+} release to a propagated E-C Coupling mode, rather than the loss of T-tubular currents, is the feature that most likely plays a major role in HCM-associated T-tubule remodelling. This slows down force development and delays the onset of relaxation. In addition, evidence is discussed that HCM-related T-tubule remodelling may contribute to the energetic defect of HCM cardiomyocytes (see also [40]).

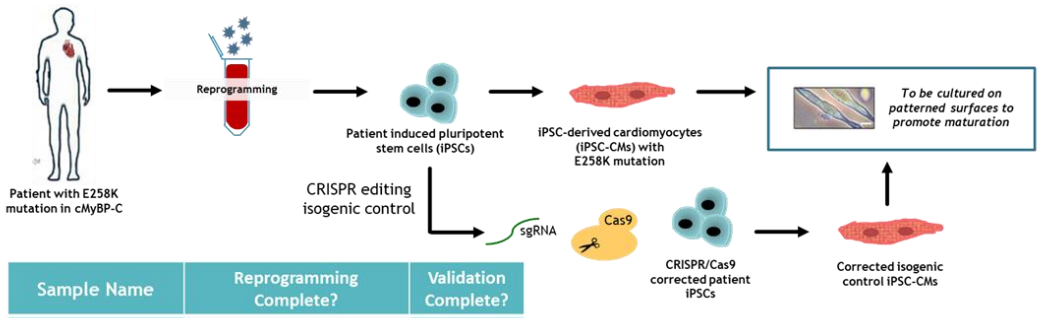
Experimental data related to the twitch contraction and E-C coupling of intact trabeculae and cardiomyocytes from HCM patients expressing the E258K mutation in cMyBPC (see also sections 2.2 and 2.5) have also been reported in the manuscript [24].

Results of the electrophysiological and E-C coupling remodelling of human HCM cardiac muscle and its impact on cardiac mechanics will be used by the MUSICO platform, implemented to simulate the cardiac action potential and intracellular Ca^{2+} cycling, besides the mechanical events in sarcomeres.

2.5 Human inducible pluripotent stem cells (hiPSCs) from patients with sarcomere protein cardiomyopathy mutations as a model system to investigate the impact of the disease at early stages on both sarcomere and excitation-contraction coupling functions.

UNIFI and UW had previously collaborated in producing in vitro models of HCM [41] and of the dilated form of Cardiomyopathy associated to Duchenne Muscle Dystrophy [42]. In the SILICOFCM project the potential of hiPSC- cardiomyocytes for investigating electrophysiology and excitation-contraction coupling in *in vitro* disease models and the implications of the advances in stem cell modelling of the Duchenne cardiomyopathy for the wider world of Dilated Cardiomyopathy have been reported and reviewed [43,44].

Following these results the collaboration between UNIFI and UW supported by SILICOFCM was extended to produce an in vitro model of the HCM disease associated with the E258K missense mutation in cardiac Myosin Binding Protein C described in Section 2.2 and representing a founder mutation in Tuscany with a significant number of patients in the Florence HCM cohort (Figure 7). To this aim UNIFI obtained peripheral blood mononuclear cells (PBMC) from 7 patients, all carrying the same E258K mutation and reported for myectomy samples in section 2.2. The PBMCs were sent to UW, where they were reprogrammed into inducible pluripotent stem cells (iPSCs). Crisp Cas 9 was used to correct the mutation for one patient line, as an isogenic control. All cell lines have been subjected to in depth validation including checks for pluripotency markers, karyotyping to confirm genetic fidelity and stability and tri-lineage differentiation potential. All cell lines have undergone directed differentiation to demonstrate that can become cardiomyocytes with long term stability in culture (Figure 13).



Sample Name	Reprogramming Complete?	Validation Complete?
Patient 1	YES	**100% complete**
Patient 2	YES	50% complete
Patient 3	YES	50% complete
Patient 4	YES	**100% complete**
Patient 4 CRISPR corrected	*CRISPR editing complete* - YES	**100% complete**
Patient 5	YES	75% complete
Patient 7	YES	50% complete

cMyBPC = cardiac myosin binding protein C
sgRNA = single guide RNA

Figure 13. In vitro human heart muscle cell models from iPSCs of E258K MyBP-C HCM patients of the UNIFI cohort.

The iPSCs were shipped to UNIFI and kept at UW for experiments. At about this time, our efforts were shut down for 2 months due to the Covid-19 pandemic. iPSCs had to be frozen during the shutdown, then had to be thawed, expanded and differentiated once labs were opened. We are now starting to get results. From the results of studies in the human myectomy samples we hypothesized that the E258K-MYBPC3 mutation cell lines would be haplo-insufficient for MYBPC3 (Figure 14). For one patient line, at multiple time points out to 60 days post-differentiation we find that indeed the MYBPC protein levels are significantly less compared with wild type and the Crispr corrected cell lines (Figure 14). This is our first and quite significant result, as it supports the haplo-insufficiency hypothesis prediction predicated from the myectomy samples. It also validates the iPSC-cardiomyocyte model as a human in vitro model to study HCM. In ongoing studies, we are culturing patient iPSC-cardiomyocyte lines for studies of contractile properties. We recently verified that myofibrils isolated from the control cell line produce similar specific force reasonable contraction and relaxation kinetics as previous studies [41,42], further validating this iPSC-cardiomyocyte model. At this time we are culturing multiple cell lines, both in Seattle (UW) and Florence (UNIFI), for measures of action potentials, calcium handling, contractile properties and contractile protein isoforms. An abstract has been submitted for the Biophysical Society meeting in February 2021 to report on these cell lines [45].

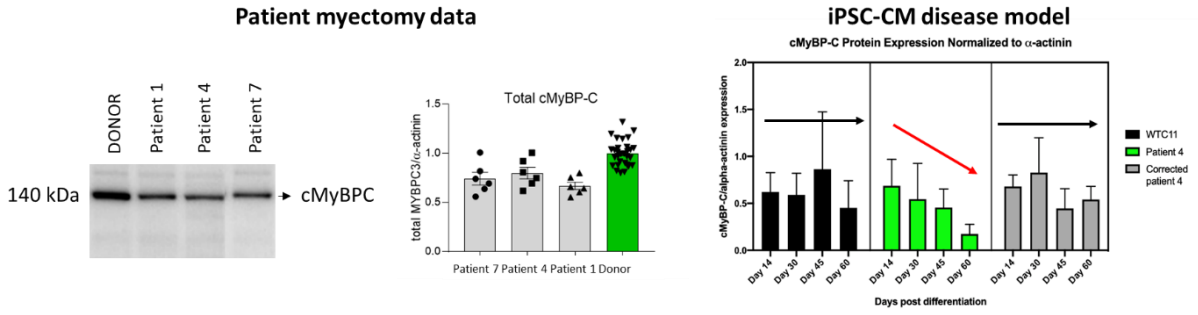


Figure 14. E258K mutation in *MYBPBC3* causes cMyBP-C haploinsufficiency in both the patient samples and in the iPSC-Cardiomyocyte *in vitro* model of the disease.

The left side of the Figure 14 shows that Haploinsufficiency is also observed during differentiation of cardiomyocytes from iPSC of patient 4; haploinsufficiency is not present following genetic correction of the cell line of the same patient. On the right side, western immunoblot using a C2C14 antibody illustrates reduced expression of full-length cMyBP-C (140 kDa) in cardiac biopsies from 3 E258K patients compared with donor tissue. In the middle, xpression of cMyBP-C is normalized to α-actinin. cMyBP-C/α-actin in donor is set to 1 (donor N=1; E258K N=3).

Once the E258K iPSC-cardiomyocyte model will be thoroughly investigated, it will also be possible to use it as a tool for drug testing.

2.6 Collaboration with WP5: Multiscale modeling of twitch contractions from human and animal models: impact of cardiomyopathy mutations and small molecules acting on the myosin motor.

2.6.1 Multiscale Modeling of Twitch Contractions in Cardiac Trabeculae.

Understanding the dynamics of a cardiac muscle twitch contraction is complex because it requires a detailed understanding of the kinetic processes of the Ca^{2+} transient, thin filament activation and the myosin-actin crossbridge chemo-mechanical cycle. Each of these steps has been well defined individually but understanding how all the three processes operate in combination is a far more complex problem. Computational modeling has the potential to provide detailed insight into each of these processes, how the dynamics of each process affects the complexity of contractile behavior and how perturbations such as mutations in sarcomere proteins affect the complex interactions of all these processes. The mechanisms involved in relaxation of tension during a cardiac twitch have been particularly difficult to discern, due to non-homogeneous sarcomere behaviour during relaxation.

Here we use the multiscale MUSICO platform to model twitches from rat trabeculae. Validation of computational models is dependent on being able to simulate different experimental data sets, but there has been a paucity of data that can provide all of the required parameters in a single experiment, such as simultaneous measurements of force, intracellular Ca^{2+} transients and sarcomere length dynamics. In this study, we used data from different studies collected under similar experimental conditions to provide information for all the required parameters. Our simulations established that multiple sarcomere trabeculae replicate the experimental observations of twitches, under either an isometric sarcomere condition or a fixed-end condition, if models incorporate a length-tension relation for the nonlinear series elasticity of muscle preparations and a scheme for thick filament regulation. The thick filament regulation assumes an off-state where myosin heads are parked onto the thick filament backbone and are unable to interact with actin, a state analogous to the super relaxed state (SRX). Including these two mechanisms provided simulations that accurately predict twitch contractions over a range of different conditions [19] (Figure 2).

Next this multiscale approach has been used to explore twitches in muscle preparations containing mutations associated with HCM & DCM – see next section 2.6.2.

2.6.2 The Effect of Variable Troponin C Mutation Incorporation in Thin Filaments on Cardiac Muscle Twitch Contractions.

One of the complexities of understanding the pathology of genetic cardiomyopathies associated to sarcomeric protein mutations is the level of mutation incorporation in sarcomeres. Computational models of the sarcomere that are spatially explicit offer an approach to study aspects of mutational incorporation into myofilaments that are more challenging to get at experimentally. To develop this approach we studied two well characterized engineered mutations of cardiac TnC (cTnC), L48Q and I61Q, that decrease or increase the rate constant (k_{-Ca}) for the release of Ca^{2+} from cTnC resulting, when expressed in the mouse heart, in animal models of HCM and DCM respectively (see Section 2.1). Expression of these mutations in transgenic mice results in incorporation of 30 and 50% respectively.

Here we demonstrate that isometric (fixed end) twitch contractions of mutant mouse trabeculae differ from WT; L48Q has a slower relaxation while I61Q has markedly reduced peak tension. Using our multiscale modelling approach developed for rat trabeculae twitches (see above), we were able to describe the tension transients of WT mouse myocardium. Tension transients for the mutant cTnCs were described with changes in k_{-Ca}, as measured in vitro for mutant cTnC, a change in the affinity of cTnC for cTnI, and a reduction in the steady state level of detached crossbridges. This reduction could be achieved in two different ways. A major advantage of our multiscale explicit 3D model is that it not only predicts the effects of variable mutation incorporation, but also the effect of variations in the way the mutant and WT TnC's are distributed throughout thin filaments in sarcomeres. Such effects are currently impossible to explore experimentally. We explored random and clustered distributions of mutant cTnC in thin filaments, as well as distributions of individual thin filaments with only WT or mutant cTnC present. The effects of variable incorporation or of non-random distribution are more marked for I61Q than L48Q cTnC [18].

2.6.3 Next Steps.

The model developed for the simulation of rat and mouse twitches will be adapted to simulate the data from human cardiac samples described in sections 2.2 and 2.4. Attention will be paid to the model predictions of conditions in which the relative expression of the fast and slow isoforms of cardiac myosin is modified [46].

The ability of the model to simulate some of the results described in Section 2.3 related to the impact of small molecules affecting the function of the myosin motor will also be tested.

Finally, electrophysiological, Ca^{2+} transient, and mechanical data from intact human cardiac preparations described in Section 2.4 and collected from the experiments described in section 2.5, will be simulated by a novel version of the MUSICO platform able to simulate the whole functional series of events starting with the action potential and ending with the mechanical response of human cardiac muscle.

3. Deviation from the work plan

The significant shift in the knowledge of the mechanisms of contraction regulation in striated muscle caused by the novel studies mentioned in Section 1.2 (i.e. the role of thick filament regulation and of the SRX state of the myosin heads) was one major reason for the accepted request of amendment for a prolongation of the timing of WP2 compared to the original proposal. Recent demonstrations of the potential of hiPSC- cardiomyocytes for investigating cardiac functional properties in *in vitro* disease models added further experimental models to the research of WP2 as it had originally been planned. WP2 experiments were delayed by several months due to the COVID-19 pandemic and the timing of the WP was additionally prolonged.

In spite of delays and prolongations, there were no major deviations in the work plan that, instead, took some quality advantages of the scientific changes described above.

4. Conclusions

4.1 Summary of achievements and key conclusions.

Achievements include:

1. A control and FCM cardiac tissue repository has been created at the Da Vinci biobank at UNIFI. This had been started during a previous EU project (Big Heart, 7th framework programme for research) and has been used, maintained, and expanded for SILICOFCM. These samples have been extensively used in WP2 and will still be used in the near future for the aims of both task 2.1 and 2.2.
2. Mechanical and energetic experiments in preparations from cardiac samples of HCM patients expressing mutations in different proteins of the sarcomere indicate that inefficient ATP utilization by myofilaments may play a central role in the pathogenesis of HCM. The use of small molecules, like mavacamten, that favour the super-relaxed (SRX) state of myosin heads may be useful to correct or prevent the problem.
3. Work in human cardiac samples and in animal models of DCM show that small molecules acting on myosin, such as Omeocamtiv and dATP, may be useful to manage diseases characterized by reduced cardiac contractility.
4. Investigations of Excitation-Contraction Coupling alterations in intact cardiac preparations from human samples of HCM patients suggest that a number of drugs affecting ion channels and signaling pathways can be used to control clinically significant features of HCM.
5. *In vitro* models of FCMs have been created by differentiating hiPSCs to cardiomyocytes. IWork started to validate the model has been successful.
6. The collaboration with WP5 has validated and extended the MUSICO modelling capability. In particular, the mouse cardiac twitch was modelled and the twitches of transgenic mice carrying mutations in TnC that either increase or decrease myofilament Ca^{2+} affinity. These mutations mimic HCM and DCM phenotypes in the mouse. The twitches were successfully modelled using just the change in Ca^{2+} affinity measured in solution.

5.2 Planned future work.

5.2.1 Complete publications.

Besides completing the publications listed in **Error! Reference source not found.** under 1a-3a, new papers are in preparation relative to the conference abstracts reported in **Error! Reference source not found.** under 1b-5b, and some additional manuscript work (both original research articles and reviews) is planned about the still unpublished achievements described in Section 2 and about the use of those achievements in WP5 to complete/improve the MUSICO platform.

5.2.2 Further studies and plans related to SILICOFCM.

Further plans include:

- Maintain and expand the FCM and control human cardiac tissue and PBMC/iPSCs repository present at the UNIFI Biobank.
- Expand the biophysical/physiological investigations on in vitro preparations from human cardiac samples of control and FCM patients carrying additional pathogenic sarcomere protein mutations and complete the investigations about the impact of potentially useful drugs in human cardiac preparations.

- Complete the experimental characterization of the E258K iPSC-cardiomyocyte model and start its use as a tool for drug testing.
- Expand the hiPSC-cardiomyocyte model to additional human HCM/FCM models.
- Utilize the power of modelling for predictions about the impact of specific HCM mutations and the impact of the heterozygous condition of HCM mutations on the function of the sarcomere.
- Contribute with experimental data to the expansion and improvement of the MUSICO platform for simulations of all molecular events involved in cardiomyocyte contraction (from the action potential to the mechanical event).

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6. Appendix 1. Publications arising from WP2 supported by SILICOFCM

Published articles supported by SILICOFCM

No	Names in bold are authors associated with SILICOFCM. Results from these publications are summarized in Section 2 of the Deliverable.
1	**Piroddi N**, Ferrara C, Witjas-Paalberends R, **Ferrantini C**, **Scellini B**, **Vitale G**, Wijnker P, Sequiera V, Dooijes D, dos Remedios C, Schlossarek S, Leung MC, Messer A, Ward DG, Biggeri A, **Tesi C**, Carrier L, Redwood C, Marston S, van der Velden J, and **Poggesi C**. The homozygous K280N troponin T mutation alters cross-bridge kinetics and energetics in human HCM. *J Gen Physiol* (**2019**) **151**:18-29. DOI: 10.1085/jgp.201812160
2	**Pioner JM**, Santini L, Palandri C, Martella D, Lupi F, Langione M, Querceto S, Grandinetti B, Balducci V, Benzoni P, Landi S, Barbuti A, Ferrarese Lupi F, Boarino L, **Sartiani L**, **Tesi C**, Mack DL, **Regnier M**, **Cerbai E**, Parmeggiani C, **Poggesi C**, **Ferrantini C**, **Coppini R**. Optical Investigation of Action Potential and Calcium Handling Maturation of hiPSC-Cardiomyocytes on Biomimetic Substrates. *Int J Mol Sci* (**2019**) **20**(15). pii: E3799. doi: 10.3390/ijms20153799.
3	**Coppini R**, **Ferrantini C**, **Pioner JM**, Lorenzo Santini, Wang ZJ, Palandri C, Scardigli M, **Vitale G**, Sacconi L, Stefàno PL, Flink L, Riedy K, Pavone FS, **Cerbai E**, **Poggesi C**, Mugelli A, Bueno-Orovio A, **Olivotto I**, Sherrid MV. Electrophysiological and Contractile Effects of Disopyramide in Patients With Obstructive Hypertrophic Cardiomyopathy: a Translational Study. *JACC: Basic to Translational Science* (**2019**) **4**(7):795-813. doi: 10.1016/j.jacbts.2019.06.004.
4	**Vitale G**, **Ferrantini C**, **Piroddi N**, **Scellini B**, **Pioner JM**, Colombini B, **Tesi C**, **Poggesi C**. The Relation Between Sarcomere Energetics And The Rate Of Isometric Tension Relaxation In Healthy And Diseased Cardiac Muscle. *J Muscle Res Cell Motil* (**2019**) Nov 19. doi: 10.1007/s10974-019-09566-2. Online ahead of print.
5	**Pioner JM**, Fornaro A, **Coppini R**, Ceschia N, Sacconi L, Donati MA, Favilli S, **Poggesi C**, **Olivotto I** & **Ferrantini C**. Advances in Stem Cell Modeling of Dystrophin-Associated Disease: Implications for the Wider World of Dilated Cardiomyopathy. *Front Physiol* (**2020**) **11**:368. doi: 10.3389/fphys.2020.00368. eCollection 2020.
6	Mazzarotto F, **Olivotto I**, Boschi B, Girolami F, **Poggesi C**, Barton PJR, Walsh R. Contemporary Insights Into the Genetics of Hypertrophic Cardiomyopathy: Toward a New Era in Clinical Testing? *J Am Heart Assoc* (**2020**) **9**(8):e015473. doi:10.1161/JAHA.119.015473
7	Powers JD, Kooiker KB, Mason AB, Teitgen AE, Flint GV, Tardiff JC, Schwartz SD, McCulloch AD, **Regnier M**, Davis J, Moussavi-Harami F. Modulating the tension- time integral of the cardiac twitch prevents dilated cardiomyopathy in murine hearts. *JCI Insight*. (**2020**) Sep 15:142446. doi: 10.1172/jci.insight.142446. Epub ahead of print. PMID: 32931484.
8	Ma W, Childers M, Murray J, Moussavi-Harami F, Gong H, Weiss R, Daggett V, Irving T, **Regnier M**. Myosin dynamics during relaxation in mouse soleus muscle and modulation by 2'-deoxy-ATP. *J Physiol* (**2020**) **598**(22):5165-5182. doi: 10.1113/JP280402.
9	McCabe KJ, Aboelkassem Y, Teitgen AE, Huber GA, McCammon JA, **Regnier M**, McCulloch AD. Predicting the effects of dATP on cardiac contraction using multiscale modeling of the sarcomere. *Arch Biochem Biophys* (**2020**) **695**:108582. doi: 10.1016/j.abb.2020.108582.

10 **Vitale G**, **Coppini R**, **Tesi C**, **Poggesi C**, Sacconi L, **Ferrantini C**: T-tubule remodeling in human hypertrophic cardiomyopathy. *J Muscle Res Cell Motil* (**2020**) Nov 22. doi: 10.1007/s10974-020-09591-6. Online ahead of print.

11 Childers MC, **Geeves MA**, Daggett V, **Regnier M**. Modulation of post-powerstroke dynamics in myosin II by 2'-deoxy-ADP. *Arch Biochem Biophys* **In Press**.

12 **S. M. Mijailovich**, M. Prodanovic, **C. Poggesi, M. A. Geeves**, and **M. Regnier** Multiscale Modeling of Twitch Contractions in Cardiac Trabeculae. *J Gen Physiol* **In Press.**

Submitted/Under revision

1 a **Vitale G**, E Lazzeri E, F Giardini F, Costantini I, **Ferrantini C**, Mazzamuto G, Bocchi L, **Piroddi N**, **Scellini B**, **Pioner JM**, Girolami F, Mazzarotto F, Pippucci T, Chiti S, Coppini R, van der Velden J, **Tesi C**, Pavone FS, **Olivotto I**, Sacconi L, **Poggesi C**: Advanced morpho-functional analysis reveals impaired cross-bridge kinetics and energetics in patients with a Myosin Binding Protein C founder mutation causing Hypertrophic Cardiomyopathy. *Circ Res* Under Revision.

2 a **Scellini B**, **Piroddi N**, Dente M, **Vitale G**, **Pioner JM**, **Coppini R**, **Ferrantini C**, **Poggesi C**, **Tesi C**. Impact of Mavacamten on force generation in single myofibrils from rabbit psoas and human cardiac muscle. *J Gen Physiol* Under Revision

3 a **S. M. Mijailovich**, M. Prodanovic, **C. Poggesi**, J. Powers, J. Davis, **M. A. Geeves**, and **M. Regnier**. The Effect of Troponin C Mutation Penetrance on Cardiac Muscle Twitch Contractions. *J Mol & Cell Cardiol* Under Review

Conference presentations

1b **Poggesi C** & UNIFI team: Advanced morpho-functional analysis on ventricular and atrial tissue reveals cross-bridge kinetics alterations and sarcomere energetic impairment in HCM patients. Motors, Myosin and Muscle, Conference Alpbach Austria, March 2019

2b **Scellini B**, **Piroddi N**, **Dente M**, **Ferrantini C**, **Coppini R**, **Poggesi C**, **Tesi C**. Impact of Mavacamten on force generation of single myofibrils from rabbit psoas and human cardiac muscle. Annual Meeting Biophys Soc San Diego, USA Feb 2020 *Biophys J*

3b **Mijailovich SM**, Prodanovic M, **Poggesi C**, **Regnier M** and **Geeves MA**. Effect of myosin isoform on mechanics in intact cardiac trabeculae from mice, rats and humans. Annual Meeting Biophys Soc San Diego, USA Feb 2020 *Biophys J*

4b **Scellini B**, **Piroddi N**, Dente M, **Vitale G**, **Coppini R**, **Ferrantini C**, **Poggesi C**, **Tesi C**. Omeocamtiv Mercabil modulation of force generation in single myofibrils from human cardiac muscle. Annual Meeting Biophys Soc, USA 2021 *Biophys J*

5b **Steczina S**, **Pioner MJ** et al: Structure-function investigations of the E258K-MYBPC3 mutation in HCM patient-derived cardiomyocytes. Annual Meeting Biophys Soc, USA 2021 *Biophys J*