SILICOFCM

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Executive summary

Inherited cardiomyopathies (Hypertrophic and Dilated CardioMyopathies, HCM, DCM) are caused by mutations in genes for proteins of the muscle sarcomere contractile apparatus. We need to know the mechanisms by which mutations affect protein behaviour and hence cardiac muscle 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 problem and prevent or manage the disease.

Few mutations have been explored at the protein, cell, organ and patient level. Therefore, a major goal of WP2 (Task 2.1 and Task 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 both validate the models and become a useful tool for predicting the effect of novel mutations. This tool will also allow us to explore the molecular and cellular details of how small molecules may modulate protein and cellular behaviour, providing potential new therapeutics.

The division between Task 2.1 (Protein and Cell Data) and Task 2.2 (Physiological experiments) is arbitrary and the two Tasks have worked very closely together throughout with free exchange of information and experience. The Deliverable reports (D2.1 and D2.2) reflect this overlap, to make clear the advantages arising from this intimate synergy, aspects of each report are duplicated where relevant and italics indicate where the D2.2 sections are reproduced here.

To complete this Task 2.1 required access to the normal human proteins/cells and the proteins/cells carrying one of the many disease linked mutations. We have obtained these using in vitro expressed human proteins or from patients' samples that include surgical tissue samples, tissue from transplanted hearts and patient blood samples that are sources to create adult stem cells that can be turned into patient-specific heart muscle cells.

In UW blood samples from UNIFI heart patients were induced into becoming Pluripotent Stem Cells (iPSC), then converted into heart muscle cells (cadiomyocytes) in cell culture. These cells carry the same mutation as the patient and allowed a study of protein expression levels, mechanics of contraction and the isolation of pure proteins. iPSCs containing a mutation in MyBPC were successfully grown and characterised.

Working with very small human samples required the development of new methodology to allow such small samples to be exploited successfully. UNIKENT developed several micro methods and used them to evaluate proteins extracted from heart samples, then applied this approach to human iPSCs. This now provides a theoretically unlimited source of patient cells for studying the disease at multiple scales of heart muscle organization and for testing and developing new therapeutic small molecules and other approaches.

These human iPSC derived cardiomyoyctes are being used to study a new paradigm for the cause of HCM, the over activation of myosin from a switched off form known as the SRX state. We have developed a method to assay the presence of the SRX in small fragments of cardiomyocytes. We have also demonstrated that this approach can be used for adult heart tissue as well as iPSCs.

Using our published data we modelled the behaviour of eight forms of human muscle myosin, using the MUSICO modelling package (WP5) to validate the modelling approach. We have further analysed seven HCM linked mutations in myosin and used MUSICO to predict the influence of the mutations on heart muscle cell contraction. Similarly, MUSICO has been used with experimental data from both proteins and intact heart cells to simulate the contraction relaxation cycle of normal heart cells and cells carrying mutations in troponin. The work demonstrates that detailed molecule measurements of mutation effects can be used to predict the behaviour of muscle cells.

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

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List of Abbreviations

Abbreviation	Explanation
CRISPER CAS 9	Crisper gene editing system
dATP	2' deoxy ATP
DCM	Dilated cardiomyoparthy
DR	Duty Ratio
FCM	Familial Cardiomyopathy
HCM fHCM	Hypertrophic Cardiomyopathy
hiPSC-CMs	hiPSCs differentiated into cardiomyocytes
hiPSCs, iPSCs	(human) induced pluripotent stem cells
Kapp	Apparent binding constant for actin
M	Myosin
Mava	Mavacamten
MUSICO	MUSICO
MyBPC	Myosin Binding Protein C
MyHC	Myosin Heavy Chain
OM	Omecamtiv mercabil
PBMCs	Peripheral Blood mononuclear blood cells
SRX	Super Relaxed State of myosin in thick filaments
Tm	Tropomyosin
Tn, TnC	Troponin, Troponin C
WT	Wild Type

1. Introduction

1.1 Modelling FCM at the molecular level as re-defined in June 2018

Many Familial CardioMyopathies (FCM), in particular Hypertrophic CardioMyopathy (HCM) and Dilated CardioMyopathy (DCM), are caused by inherited mutations in sarcomeric proteins Myosin (M), Myosin Binding Protein-C (MyBP-C [1]) Troponin (Tn), Tropomyosin (Tm). We need to know what the mutations do to change the protein behaviour and hence muscle contraction and how adaptation to these changes results in disease. If we understand the problem, then we can ask if small molecules can be used to correct the problem and prevent or manage the disease. For example, do all HCM linked mutations cause the same response in the cell which leads to the disease or is there a different set of responses i.e., there are multiple forms of HCM?

Currently we do not know enough about the molecular details to model the system effectively. Few mutations have been thoroughly explored at the protein, cell, organ and patient level. The role of WP2, therefore, was to explore the mutations in the cardiac system at the molecular and cellular level, to build the data into appropriate detailed models (with WP5), to thoroughly validate the models and then use the models to make predictions about novel mutations. At the same time, we explore the molecular and cellular details of how small molecules may modulate protein and cellular behaviour providing potential therapeutics. The studies themselves are large long-term complex studies which will continue long after the end of this SILICOFCM project. The role of WP2 and the research groups was therefore to provide the most up-to-date information on molecular, cellular and functional studies to ensure that the design of the modelling software was appropriate.

To complete this task required access to the normal human proteins/cells and the proteins/cells carrying one of the disease linked mutation. We can obtain these from model systems (using in vitro expressed human proteins or from hearts of animal models), or from patients samples (surgical tissue samples, transplanted hearts or induced Pluripotent Stem Cells (iPSCs) derived from patient samples). Each of these has its advantages and limitations. We are working with each of these systems exploring which is the best way to obtain the specific information required. Each will be introduced in context.

Here we present Deliverable 2.1 which deals with data collected at the molecular and cellular level. The partner Task 2.2 deals with the same information in terms of physiology and is presented in D2.2. The two partner WPs 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. Where this occurs, it is indicated by cross reference to the relevant section in D2.2 and appears in italics.

1.2 The SRX

There has been a significant shift in the view of the causes of familial Hypertrophic CardioMyopathy since the SILICO grant application was assembled. Data accumulated over the last 5 years suggests that ~40% of inherited HCM cases carry a mutation in the major thick filament protein, myosin, and a further ~40% in the thick filament regulatory protein myosin binding protein C (MyBP-C). In each case at least half of the mutations are now thought to have their effect by increasing the availability of myosins by altering the thick filament off-state (variously called the super relaxed state, the interacting heads state or the J-motif) [2-5]

If this hypothesis is proved correct this SRX state not only provides a novel explanation for a major proportion of the HCM cases but also a new potential drug target. Drugs targeted at down regulation of the active fraction of myosin heads could correct the problem caused by the initial mutation. One such drug, Mavacamten has already completed stage 3 clinical trials and is recommended for treatment of certain classes of HCM [6].

We now need to build a thick filament regulation model into the current MUSICO program (WP5). To do this we need an experimental strategy to define the parameters of the thick filament regulation, including identifying mutations that operate primarily via thick filament regulation. Such data needs to be built into the MUSICO program, using experimentally defined model parameters and then testing the model predictions experimentally.

1.3 Potential small molecule drugs

Work on three small molecules as potential drugs targeted at β-cardiac myosin have been published during the course of this Task; 2’ deoxy-ATP (dATP), Mavacamten (Mava), Omeocamtiv Mercarbil (OM). Two of these have now completed stage 3 trials and the UNIFI group was the EU coordinating center of one of the Mava trials [6,7]. In addition, the UW group has been the prime mover in the development of dATP as a potential therapeutic agent [8]. We have published studies of two of these drugs as part of the SILICOFCM funded work (Table 1 P7 & P9).

1.4 Induced Pluripotent Stem Cells from human patients with FCM (Collaboration with Task 2.2, see D2.2 section 1.4 and 2.5)

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 blood cells (PBMCs) from 7 patients with a de novo mutation in myosin binding protein C (MyBP-C) that results in HCM. PBMCs were shipped to UW where they were genetically reprogramed to become human inducible pluripotent stem cell lines that are subsequently differentiated into cardiomyocytes (iPSC-CMs). Gene editing via CRISPR Cas9 has been performed on one patient iPSC 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 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. This work is reported in Section 3.1 and Figure 9. Parallel studies have begun on patient myectomy samples at INIFI (within Task 2.2) and the same patient's derives iPSCs in UW (within Task 2.1). This section is reproduced in both deliverable reports (D2.1 and 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). The MUSICO package included units to model (i) the crossbridge cycle its regulation in solution, (ii) contraction of a single sarcomere (iii) whole myocyte 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 analyse data (Section 2.4 and Figure 7) and data from the WP2 members has been used to develop MUSICO packages for the modelling of cardiac twitch contractions in normal myocytes and in those carrying, mutations in the sarcomeric proteins (Section 4, Figure 10-13). Section 4 describes the jointly published work of WP2 and WP5 that includes major contributions within Task 2.1. Proteins and Cell Data and Task 2.2, Physiology Data. This part of the report appears in both D2.1 and D2.1.

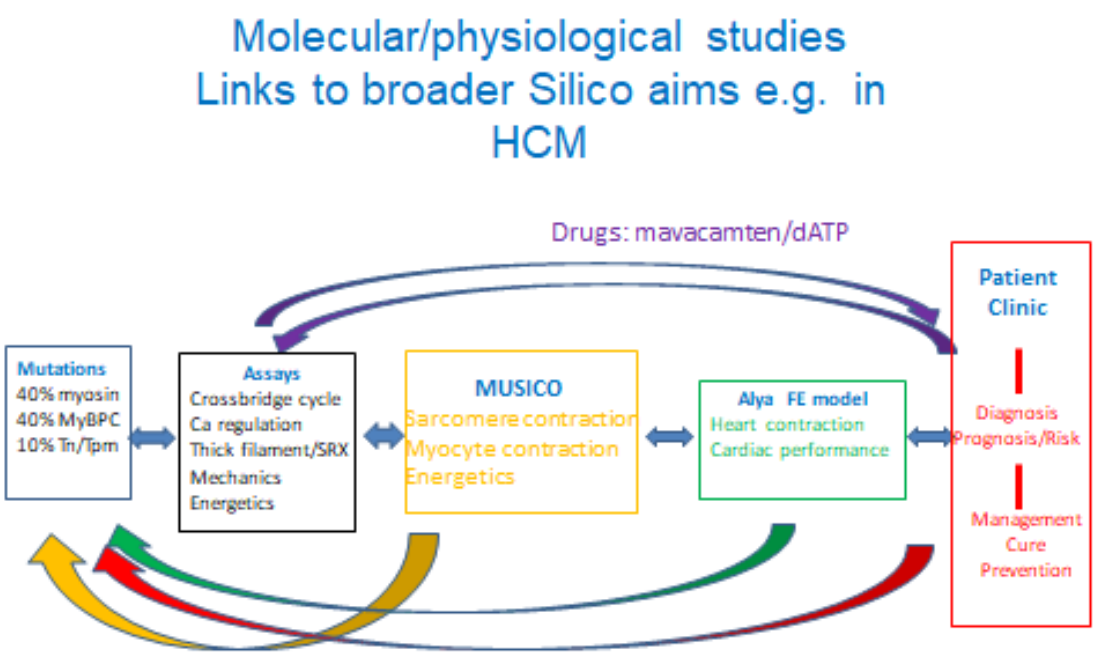


Figure 1. Relationship of WP2 (blue /black boxes) with the broader SILICOFCM project and the modelling packages of WP5 (yellow/green boxes).

The double headed arrows indicate the two-way exchange of information between each level of the study from the molecule studies of the mutations on the left (Task 2.1) 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 cellular studies and the patient data emphasised by parallel studies of therapeutic small molecules.

1.6 Published outputs

The studies completed in Task 2.1 have in most cases been published (Table 1). The findings of each publication are summarized in Sections 2 - 4 below. In a few cases the work is incomplete and will continue beyond the end of the SILICOFCM project. These are outlined also in sections 2 - 4 and the future plans are listed in Section 6.2.

Table 1. Publications arising from Task 2.1 supported by SILICOFCM.

No	Publication (Names from UNIKENT, UW, UNIFI, IIT and BIOIRC in bold are authors associated with SILICOFCM)
P1	Nebulin and Titin Modulate Crossbridge Cycling and Length Dependent Calcium Sensitivity **Srboljub Mijailovich, Boban Stojanovic, Djordje Nedic,** Marina Svicevic, **Michael Geeves**, Thomas Irving, and Henk Granzier (2019), J General Physiol details 151 (5), 680-70 DOI: 10.1085/jgp.201812165
P2	Johnson C, **Walklate J**, Svicevic M, **Mijailovich S**, Vera C, Karabina A, Leinwand L & **Geeves M**. Journal of Biological Chemistry (2019) 294(39) 14267-14278. The ATPase cycle of human muscle myosin 2 isoforms: adaptation of a single mechanochemical cycle for different physiological roles. DOI: 10.1074/jbc.ra119.009825.
P3	**Walklate J**., Ujfalusi, Z., Behrens, V., King, EJ, & **Geeves, MA**. 2019 A micro-volume adaptation of a stopped-flow system; use with microgram quantities of muscle proteins. Anal Biochem; 581: 113338. https://doi.org/10.1016/j.ab.2019.06.009
P4	Vera, C.D., Johnson, C.A., **Walklate**, **J**., Adhikari, A., Svicevic, M., **Mijailovich**, **S.M**., Ariana C. Combs, A.C., Langer, S.J., Ruppel, K.M., Spudich, J.A., Leinwand, L.A., and **Geeves, M.A.,** J. Biol Chem 294, 17451-17462. Myosin motor domains carrying mutations implicated in early or late onset HCM have similar properties. doi: 10.1074/jbc.RA119.010563. First published BioRxiv April 30th 2019
P5	Doran MH, Pavadai E, Rynkiewicz MJ, **Walklate J**, Bullitt E, Moore JR, **Regnier M, Geeves MA**, Lehman W. Cryo-EM and Molecular Docking Shows Myosin Loop 4 Contacts Actin and Tropomyosin on Thin Filaments. Biophys J. 2020 119:821-830. doi: 10.1016/j.bpj.2020.07.006. PMID: 32730789; PMCID: PMC7451941.
P6	**S. M. Mijailovich**, **M. Prodanovic**, **C. Poggesi, M. A. Geeves**, and **M. Regnier** Multiscale Modeling of Twitch Contractions in Cardiac Trabeculae. J Gen Physiol published on line Jan 29th 2021. Full pulication in vol. 153 no. 3, March 2021
P7	M.C. Childers, **M. Geeves**, V. Daggett, **M. Regnier**, Modulation of postpowerstroke dynamics in myosin II by 2'-deoxy-ADP, Archives of Biochemistry and Biophysics (2021), doi: https://doi.org/10.1016/j.abb.2020.108733.
P8	**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. Mol & Cell Cardiol. In revision.
P9	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.

2. Protein data

2.1 Sources of human myosin

2.1.1 C2C12 mouse cell line

Mammalian muscle myosin cannot be expressed in any of the standard protein expression systems (E.coli, insect cells, Hela cells) because they lack essential chaperones for correct folding of this complex structure [9]. Winkelmann [10] pioneered the use of mouse C2C12 cells in culture, differentiated into myoblasts as a suitable cell line to express human myosin. With the Leinwand lab in Colorado we have exploited this approach to express mg quantities of all 8 major human muscle myosins isoforms [11,12]. In the SILICOFCM project we have used

a. data previously published on these isoforms with MUSICO to define how each isoform is adapted for specific function (Table 1, P2)
b. previously published data on expressed human β-cardiac myosin expressing DCM linked mutations with MUSICO [13].
c. Expressed a set of 8 HCM mutation associated with earl or late onset HCM analysed the crossbridge cycle and modelled in MUSICO (P4)

2.1.2 Isolation of myosin from human tissue

We have developed the protocols for isolating myosin and subfragment 1 from frozen cardiac tissue. These have established that we can isolate 2-3 mg of functional myosin from 100 mg of tissue or 0.1 mg of the functional fragment of myosin S1. (Figure 2A). Similarly, we can isolate myofibrils from 10 mg frozen tissue and using fluorescently labelled ATP we can assess key events in the cross-bridge cycle (Table 1, P3). These assays have been used in proof of principle measurements of samples from the Florence tissue bank. These include samples from unused transplant hearts (*normal*) and human patients undergoing myectomy (Figure 2B).

Dr Walklate spent 1 week in Florence learning how to prepare myofibrils from human tissue. Human cardiac samples were then shipped from UNIFI (DaVinci biobank) to Kent to begin the analysis of cardiac tissue from control and from HCM human patients. This work is ongoing.

The samples available are small and valuable. We have therefore modified our equipment to allow a 4 fold reduction in the quantities of protein or myofibrils that will be need per experiment. This will ensure we make maximum use of the human sample was available (see Section 2.2.1 and Table 1 P3).

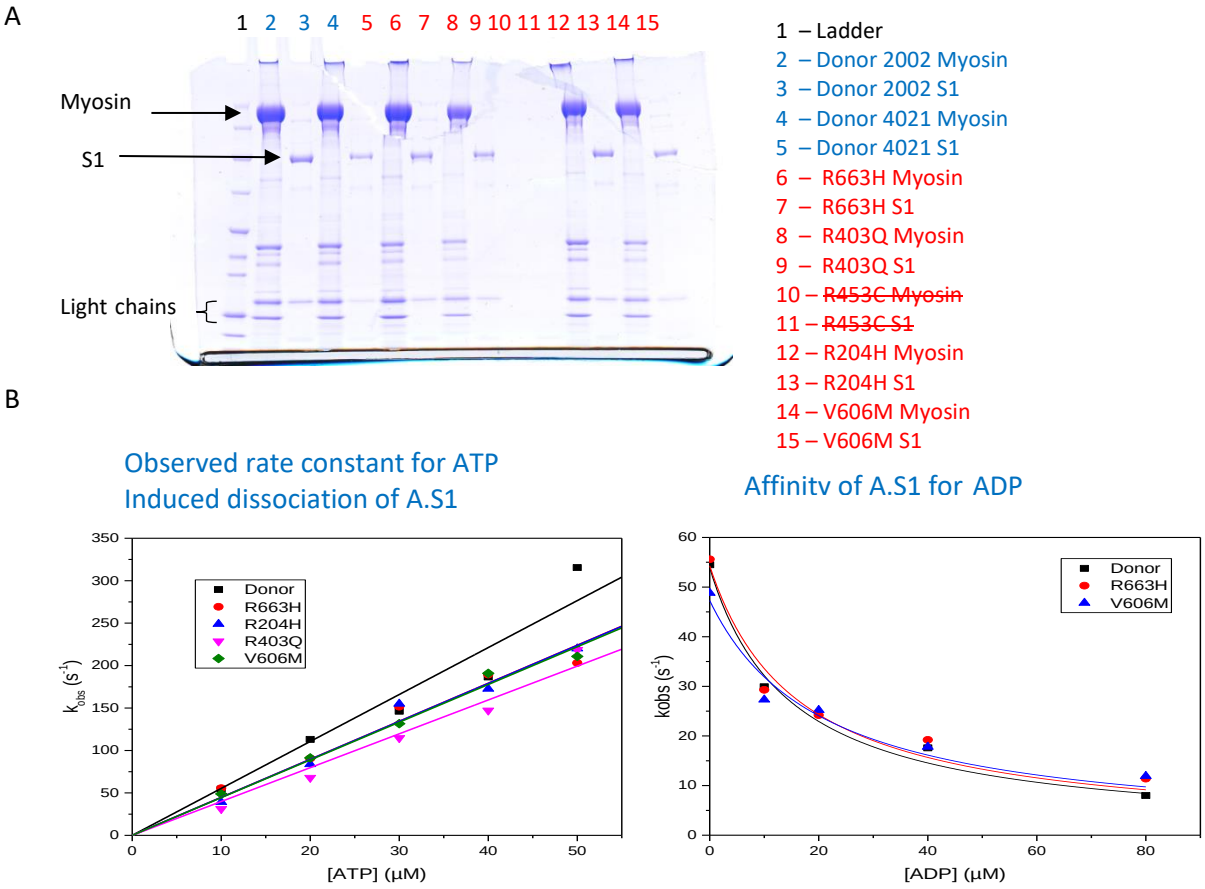


Figure 2. Isolation purification and analysis of myosin and myosin subfragment 1 from human heart biopsies. The tissue was supplied by UNIFI and the protein prepared in UNIKENT.

A) SDS-PAGE of the purified samples from 14 patients. Normal donor samples are in blue. HCM patients undergoing myectomy are in red. Samples 10 & 11 yielded no protein. Average tissue sample size 60 mg (range 20-120 mg). Average yield of myosin 1.3 mg. Ave. yield of S1 0.1 mg. Sample quality is variable as expected from variations in original samples (patients differ, sample age, time before freezing for storage). B) Stopped flow analysis of 0.1 mg of S1 measuring the rate at which ATP dissociates an actin. S1 complex and the affinity of ADP for actin.S1. Data shown for donor sample and 2-4 mutations.

2.1.3 Myosin from human iPS Cells

Myosin was extracted from wild the iPS Cells generated in UW were used by both UW and UNIKENT to extract and purify human myosin to demonstrate that such an approach is viable. The yield of myosin was typically 0.5 mg myosin from 40 x10^6 cells (Figure 3). At UW myosin was used in motility assays which measured the speed at which the myosin attached to a surface can move actin filaments. At UNIKENT the myosin was used to perform stopped flow assays of ATP dissociation of actin.myosin with the micro volume system (see Section 2.2.1 and Table 1 P3). Both demonstrated that the myosin was viable and indistinguishable from the myosin from C2C12 cells of from biopsies. This established that it will be possible to extract and characterise myosin carrying mutations from iPS cell from patents carrying FCM mutations in their myosin. The iPSC approach has been used more extensively by UW and UNIFI to study the cohort of patients from Tuscany carrying a mutation in MyBP-C (see Section 3.1 and D2.2).

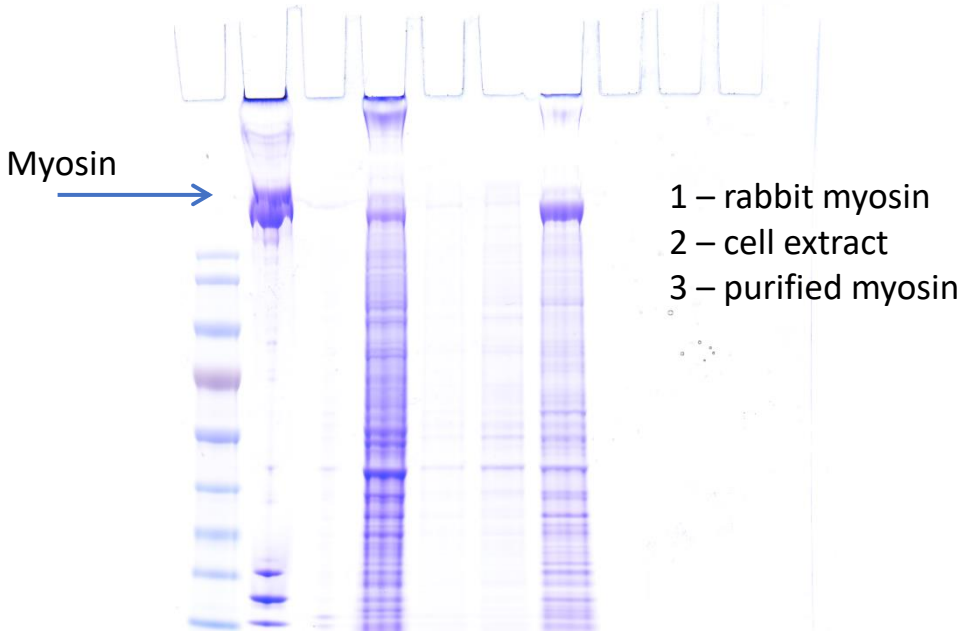


Figure 3. SDS- PAGE of Myosin purified from iPSCs.

The cells are induced to skeletal muscle cells for 60 days before harvesting. The patients' cells are supplied by UNIFI, cells are grown and transformed into muscle cells at UW and then myosin isolated at UNIKENT. UW supplied ~40 x106 cell. Yield of myosin ~0.5 mg.

2.2 New methods protein assay methods

2.2.1 Microvolume SF system (Table 1, P3)

One of the problems with experimental work using protein derived from patients carrying a FCM mutation (from biopsies or patient derive iPSCs) is the quantities of protein are very limited. To address this, we built a modified stopped flow system to reduce the volume of sample required from 500 to ~ 120 µl. With this adaptation we show that many standard myosin-based assays can be performed using <100 µg of myosin (Figure 4). This adaptation both reduces the volume and therefore mass of protein required and also produces data of similar quality to that produced using the standard set up. The 100 µg of myosin required for these assays is less than that which can be isolated from 100 mg of muscle tissue. With this reduced quantity of myosin, assays using biopsy samples become possible. This will allow assays to be used to assist diagnosis, to examine the effects of post translational modifications on muscle proteins and to test potential therapeutic drugs using patient derived biopsies or iPSCs. In addition, the system can be used with myofibrils derived from biopsies or from iPSCs (see P3 and Section 2.3, Figure 5 and Figure 6).

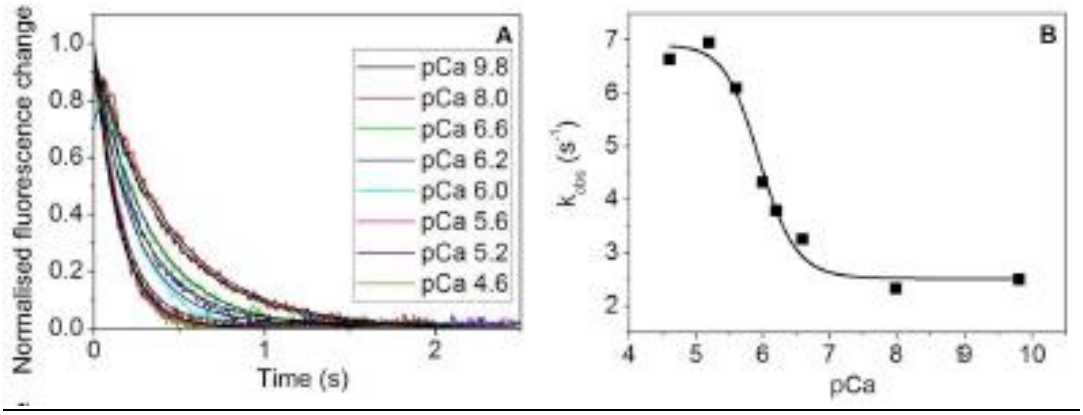


Figure 4. Ca^{2+} dependence of myosin S1 binding to thin filaments.

0.25 µM Rabbit Psoas S1 was rapidly mixed with 10-fold excess pyrene-labelled actin incubated with 2.5 µM skeletal control proteins at different calcium concentrations. (A) Averaged traces from a series of pCa concentrations. These were best described by a single exponential which are shown with a solid line. These traces have been normalised to highlight that the rate constant of S1 binding increases at higher calcium concentrations. (B) A Hill coefficient plot of the observed rate constant versus the pCa. This gave a pCa50% value of 5.95 which is in agreement with the published value of 6.02 [26]. Data collected using 35 µg of myosin S1. From P3 Fig. 6.

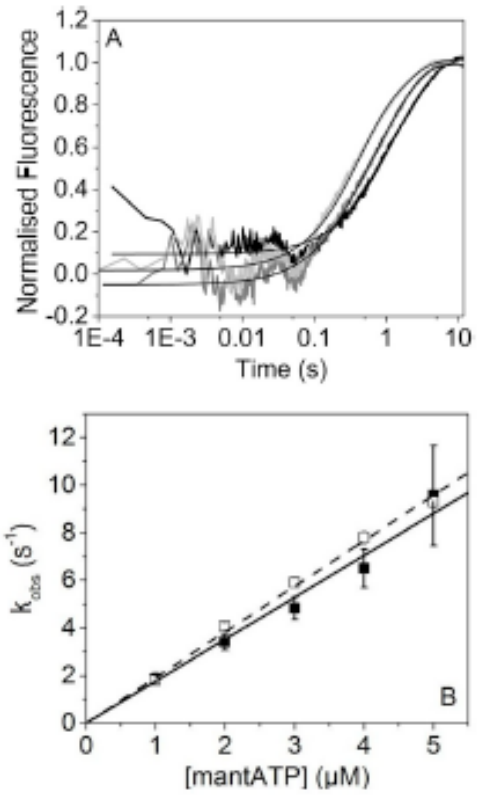


Figure 5. Mant ATP binding to 200 nM Mouse *Gastrocnemius* myofibrils.

200 nM Mouse *Gastrocnemius* Myofibrils were rapidly mixed with varying concentrations of mantATP. (A) Average traces obtained by mixing myofibrils with varied mantATP concentrations (1–5 μM) using the MVM. These were best described by a double exponential fit. The traces shown are 2 μM (black line), 3 μM (dark grey line), and 4 μM (light grey line) mantATP. These traces have been normalised to highlight that there is an increase in k_{obs} as the mantATP concentration increases. (B) A linear regression plot of the k_{obs} at mantATP concentrations 1–5 μM. The standard stopped-flow set-up gave a K_1k_{+2}=1.76 ± 0.07 μM^{-1} s^{-1} (closed squares, solid line) while the microvolume unit set-up gave a K_1k_{+2}=1.92 ± 0.03 μM^{-1} s^{-1} (open squares, dashed line). Error bars are standard error of the mean. Data collected using 270 μg of myofibrils. From P3 Fig 7

2.2.2 Quenched flow ATPase assays

UW and UNIKENT have explored the use of HPLC & Mass Spectrometry for the detection of nano-moles of ATP. This assay, developed at UW, is a potential an assay tool for use with human cardiac myosin ATPases and the detection of the rapid burst of phosphate. Such an assay can be used to examine the fraction of myosin in the off (or super relaxed) state. The miss-regulation of the off-state is a current focus of attention for explaining > 50% familial HCM cases. This is ongoing and following the shipping of 2 sets of experimental samples to UW for analysis, the approach appears to have promise. It may allow detailed assays of the small amounts of ATP consumed by human cardiac myosin derived from either stem cells or human biopsies.

2.3 The SRX

We have explored two approaches to define the SRX.

1. Using bulk fluorescence with the 2-headed fragment of myosin HMM
2. Using myofibrils and fluorescence imaging – both standard wide field imaging of myofibrils and single molecule approaches.

We have also started testing the impact of mavacamten (a drug targeted at increasing the fraction of myosin heads in the super relaxed state) on different myofibril models.

The super relaxed state (SRX) was discovered by measuring the rate at which a fluorescently labelled ATP (Fl-ATP) is turned over by myosin in an isolated or synthetic thick filament by chasing the ATP off with a large excess of unlabelled ATP. A technical problem with this approach is that is requires the sample to be mixed twice once to with the Fl-ATP and then again to chase with unlabelled ATP. This provides a challenge when the normal relaxed myosin head turns over ATP within a few seconds and the SRX turns over in >100 sec. both time scales are difficult to capture. We have adapted a standard double mixing stopped flow system to address this problem added to our micro-volume system (section 2.2.1) this allows us to assess the SRX in biopsy samples. To do this we have used myofibrils, small fragments of muscle consisting of ~ 20 sarcomeres (~40 µm long <5 µm dia). Rigor myofibrils were mixed with 10 µM mantATP incubated for 2 sec and then mixed with a large excess of unlabelled ATP. The ensuing fluorescent transient has two components a fast phase (0.02-0.05 s^{-1} representing relaxed, RX, myosin heads) and a slow phase (~0.005 s^{-1} representing SRX heads). The ratio of the two amplitudes is proportional to the number of heads in the DRX and SRX state (see Figure 6A). Low temperature is known to reduce the number of SRX heads while the drug mavacamten increases the number of heads in the SRX state (see Figure 6B). This novel assay will now allow us to assess the level of the SRX state in myofibrils from human biopsies or from iPSCs.

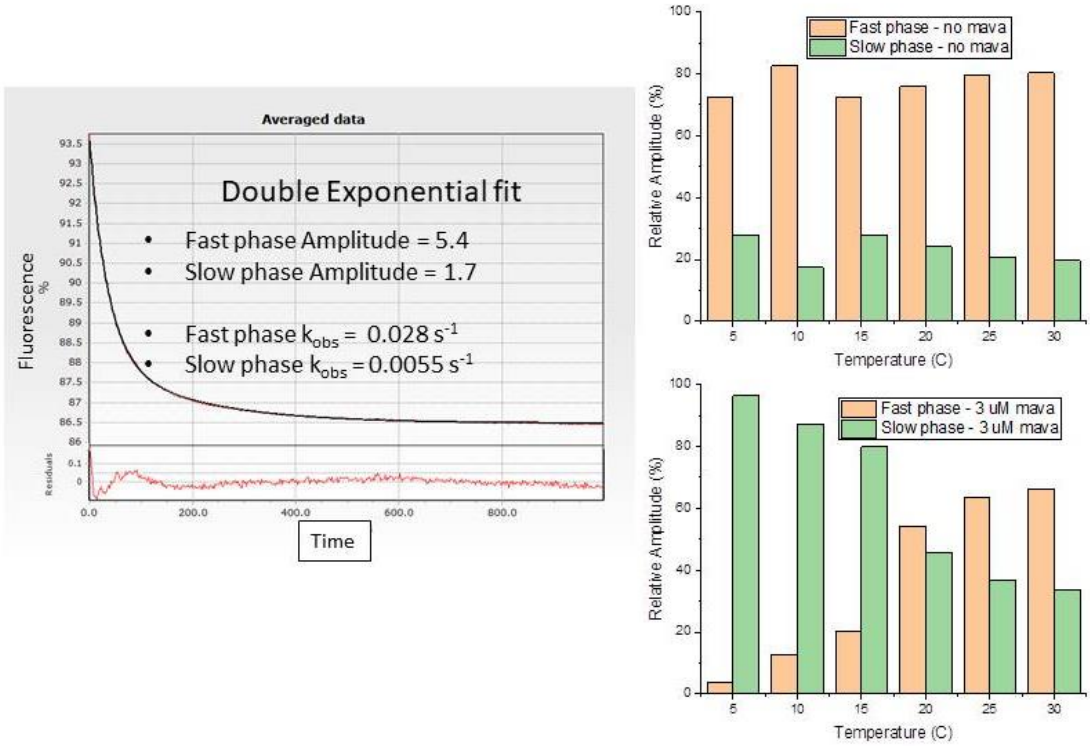


Figure 6. Analysis of the SRX in myofibrils using a double mixing stopped flow assay.

A) 200 nM myofibrils were mixed with 2 µM fluorescent mantATP and allowed to incubate for 2 sec before being mixed with a large excess of unlabelled ATP. The transient shows 2 phases a fast phase at 0.028 s-1 (the rate of mantATP turnover by myosin), and a slow phase at 0.0055 s-1 (the rate at which the super relaxed state of myosin turns over mantATP). The relative amplitudes of the two phases are proportional to the fraction of myofibril myosins in the active and super relaxed state. B) A plot of the fast (orange) and slow phases of mantATP turnover as a function of temperature in the absence (upper panel) and presence of 3 µM mavacamten (lower panel). In the absence of mavacamten the fast phase predominates ~70-80% of the total and is independent of temperature. In the presence of mavacamten there is a strong temperature dependence. At 5 °C the slow phase dominates ~95% and this decreases to ~35% at 30 °C.

2.4 HCM & DCM mutations analysed at the protein level

2.4.1 Early vs late onset HCM mutations (Table 1, P4)

Hypertrophic cardiomyopathy (HCM) is a common genetic disorder characterized by left ventricular hypertrophy and cardiac hyper-contractility. Mutations in the β cardiac myosin heavy chain gene (β-MyHC) are a major cause of HCM, but the specific mechanistic changes to myosin function that lead to this disease remain incompletely understood. Predicting the severity of any β-MyHC mutation is hindered by a lack of detailed examinations at the molecular level. Moreover, since HCM can take ≥20 years to develop, the severity of the mutations must be somewhat subtle. We hypothesized that mutations that result in early onset disease would have more severe changes in function than do later onset mutations. Here, we performed steady-state and transient kinetic analyses of myosins carrying one of seven missense mutations in the motor domain. Of these seven, four were previously identified in early onset cardiomyopathy screens. We used the parameters derived from these analyses to model the ATP driven cross-bridge cycle using MUSICO. Contrary to our hypothesis, the results indicated no clear differences between early and late onset HCM mutations. Despite the lack of distinction between early and late onset HCM, the predicted occupancy of the force-holding actin.myosin.ADP complex at [Actin] = 3 K_{app}, along with the closely related duty ratio (DR; the fraction of myosin in strongly attached force-holding states) and the measured ATPases all changed in parallel (in both sign and degree of change) compared to wild type (WT) values. Six of the seven HCM mutations were clearly distinct from a set of previously characterized DCM mutations all of which had impaired function.

2.5 Small molecule modulators of muscle function

2.5.1 Modulation of post-power-stroke dynamics in myosin II by 2'-deoxy-ADP (Table 1, P7)

Myosins generate mechanical work by hydrolysing ATP, through coordinated interactions with actin filaments. During this cross-bridge cycle, functional sites in myosin 'sense' changes in interactions with actin filaments and active site ligands, resulting in allosteric transmission of information throughout the structure. This is the structural basis of the dynamic cross-bridge cycle, which can be modulated in a nucleotide dependent fashion. We hypothesized that unique catalytic substrates yield distinct dynamics through modulation of allosteric communication pathways. We tested this hypothesis with molecular dynamics simulations of the myosin II motor domain from *Dictyostelium discoideum* in the pre- and post-power stroke states of the cross-bridge cycle, comparing ADP versus 2'-deoxyADP. We found that replacement of ADP with dADP in the post-power stroke state shifts the

conformational distribution of myosin heads toward a structure with properties that favor actin binding. Our results provide atomic level insights into allosteric communication networks in myosin that provide insight into the nucleotide-dependent dynamics of the cross-bridge cycle and may provide insights into how dATP alters the mechanochemical crossbridge cycle. dATP has been proposed as a potential therapeutic agent capable of increasing the power output of cardiac muscle – Section 3.2.

2.6 Cryo EM of actin myosin (Table 1, P5)

A major limitation in attempts to understand how point mutations in myosin generate FCMs is the absence of a high-resolution structure of the actomyosin interface. We have crystal structures of cardiac myosin motor domain and of F- actin but although structures of non-muscle myosin bound to actin have been resolved we are missing details of any actin myosin interface using a skeletal or cardiac muscle. In December of 2019 we initiated a collaboration with the group of W. Lehman (Boston University) to resolve the structure of the actin myosin complex using cryo-EM. UNIKENT supplied purified MyHC-7 β-myosin motor domain from bovine tissue and is 98% identical to the human isoform. This is the isoform found in both ventricle and slow skeletal muscle. The Boston group collected cryo-EM data of the actin myosin complex and the data was published in the Biophysical Journal this summer (P5). The results show both the first images of the actin myosin interface at < 10 Å resolution for a mammalian β myosin and for the first time the details of the interface between tropomyosin actin and myosin in the rigor complex. This will allow better understanding of FCM mutation that affects this interface.

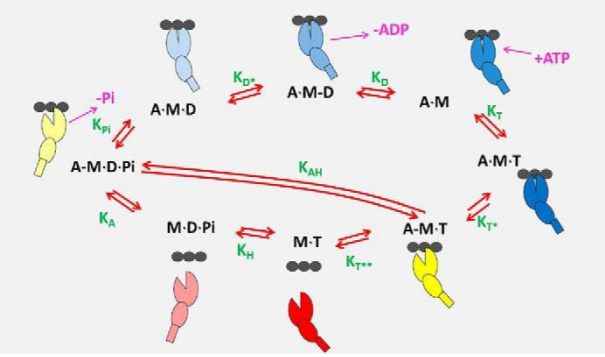
2.7 Validation and extension of MUSICO (Publications 2 & 4)

In the year before commencement of the SILICOFCM project we had worked with Dr Mijailovich from IIT to develop and use the MUSICO package to model the crossbridge cycle in solution of purified proteins. Using data the Kent group had previously collected on the human myosin isoforms we published two papers; the first used MUSICO to predict the state occupancies of the crossbridge cycle for the two human cardiac myosin isoforms α and β and compared the predicted cycles with that of the classic fast muscle myosin from rabbit [14]. The second used MUSICO to analyse the crossbridge cycle of β-cardiac myosins carrying six distinct mutations linked to DCM [13]. Each mutation altered the cycle in different ways but in each case the analysis showed the myosin was predicted to have a reduced force generating capacity – a characteristic of the hypocontractile DCM phenotype. In the first year of the SILICOFCM project we extended the work of both of these ground breaking papers.

The work on the α and β cardiac isoforms showed that each isoform had a distinct pattern of occupancy of the major states of the cycle consistent with what is known about the characteristics of contraction of the two types of cardiac muscle. As a test of the power of the MUSICO package we extended the analysis as part of SILICOFCM to all major eight human myosin isoforms of skeletal and cardiac muscle (α, β, IIA, IIB, IIx, extraocular and the two developmental isoforms embryonic and perinatal) and broadened the predictions to include economy of ATP usage by each isoform as well as maximum velocity of contraction and the load dependence of the cycle (see Figure 7 and P2). Again, each isoform had a distinct pattern of predicted behaviour consistent with the known contractions of muscle fibres expressing solely these isoforms. This is important because it begins to explain why the same mutation in the different myosin isoforms has different phenotypes.

The work on the DCM linked mutations suggested the MUSICO modelling based on detailed experimental definition the crossbridge cycle could predict contractile characteristics of each mutation. Next, we examined 7 HCM linked myosin mutations 3 associated with adult onset of HCM and 4 with early onset Section 2.4.1. The assumption being that the early onset phenotype may be linked with more severe changes to the myosin.

A)



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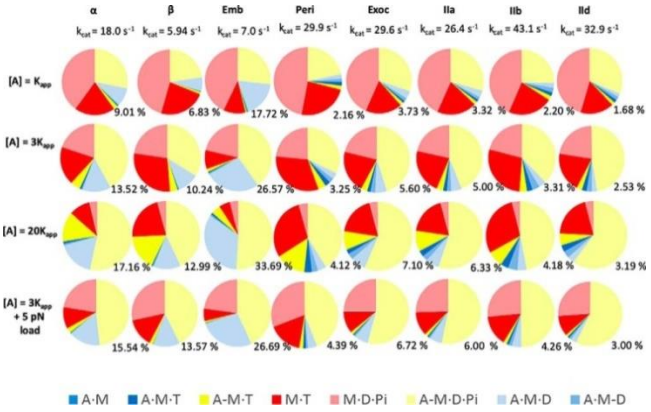

C)

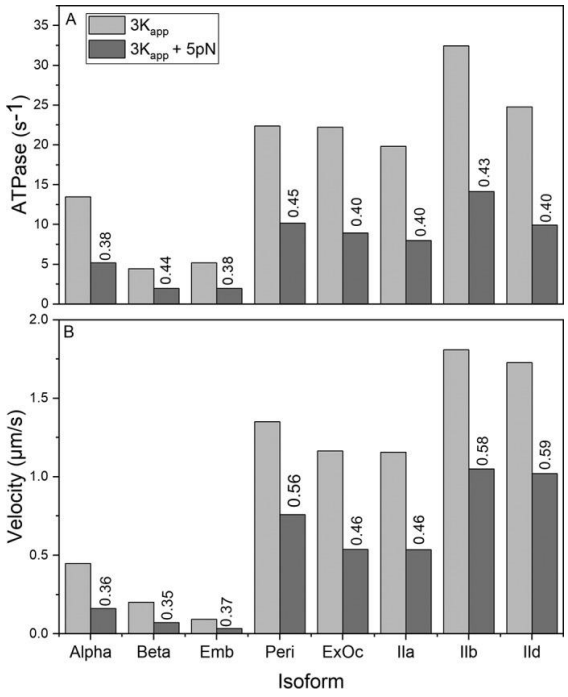

Figure 7. Modelling the crossbridge cycle for eight isoforms of human muscle myosin.

A) Eight state model of the crossbridge cycle implemented in MUSICO. B) Fractional occupancies of each state in the ATPase cycle at three different actin concentrations, [A] = K_{app}, $3K_{app}$, and $20K_{app}$, for each isoform and at [A] = $3K_{app}$ plus 5-pN load. Colors of the pie chart match those in A. The percentage value next to each chart gives the percentage of each cycle spent as the force-holding A·M·D state. C) Predictions of the ATPase rate and velocity of shortening for each myosin isoform.

3. Cell data

3.1 Human inducible pluripotent stem cells (iPSCs) as a model system to investigate the impact of the disease on sarcomere function

We previously obtained peripheral blood mononuclear cells (PBMC) from 7 patients, whom are reported for myectomy samples in points 1 and 3, with the E258K-MYBPC3 mutation. The PBMCs were reprogrammed into inducible pluripotent stem cells (iPSCs). Crisper 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. About this time, our efforts were shut down for 2 months due to the Covid-19 pandemic. iPSC were frozen, then had to be thawed, expanded and differentiated once labs were opened.

In ongoing studies, we are culturing patient iPSC-cardiomyocyte lines for studies of contractile properties. UNIFI and UW have pioneered the approach to isolate and make mechanical measurements from myofibrils of iPSC-CMs. Figure 8B & C demonstrates the force trace data that can be obtained (left - adult rat cardiac myofibril; right – myofibril from patient iPSC-CM containing the E258K mutation).

A custom-built apparatus allows rapid changes from relaxation to activation solutions, and back, independent of the Ca^{2+} transient dynamics of intact muscle cells to assess the properties of the contractile organelles, which are influenced by protein isoforms [15], Figure 8A. We will measure the rate of contractile activation (k_{ACT}) and both the early, linear slow phase (k_{REL}, slow) and later, fast phase (k_{REL}, fast) of relaxation. k_{ACT} measures the rate of myosin binding to actin and subsequent force generation and depends on myosin binding kinetics and crossbridge cycling rate. k_{REL}, slow measures the rate of myosin detachment and the length of the linear phase ($t_{REL,slow}$) is the time it takes thin filaments to deactivate [16]. These measures will be made in normal (wild type), patient (E258K cMyBP-C) and patient isogenic cell lines (see Figure 8).

An important question in understanding the mechanism of the disease for this mutation is whether the contractile abnormalities result from altered interaction of the protein containing the mutant (cMyBP-C) or if the mutation in the protein leads to it being degraded and metabolized in cells, resulting in a loss of protein in the myofibril contractile element. This latter condition is called haploinsufficiency and the UNIFI group has reported that this is what is seen in heart (myectomy)

samples from patients. This is shown in Figure 9 (left and center panel). As a first major finding for our patient stem cell lines, our data to date suggest the same thing occurs with the hiPSC-CMs. The right panel of the figure shows that over a range from 14 to 60 days after differentiation of stem cells into cardiac myocytes, the level of cMyBP-C stays relatively constant in control (WTC11) cells and in the isogenic control cells (Corrected, patient 4), while the level is similar early on in patient hiPSC-CMs but diminishes over time and is significantly less by day 60. We are currently increasing our number of replicates to verify this, which would help validate that these hiPSC-CMs can be used to model human HCM.

At this time, we are culturing multiple cell lines, both in UW and UNIFI, for measures of 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.

We recently verified that myofibrils isolated from the control cell line produce similar specific force reasonable contraction and relaxation kinetics as previous studies [17, 18], further validating this iPSC-cardiomyocyte model.

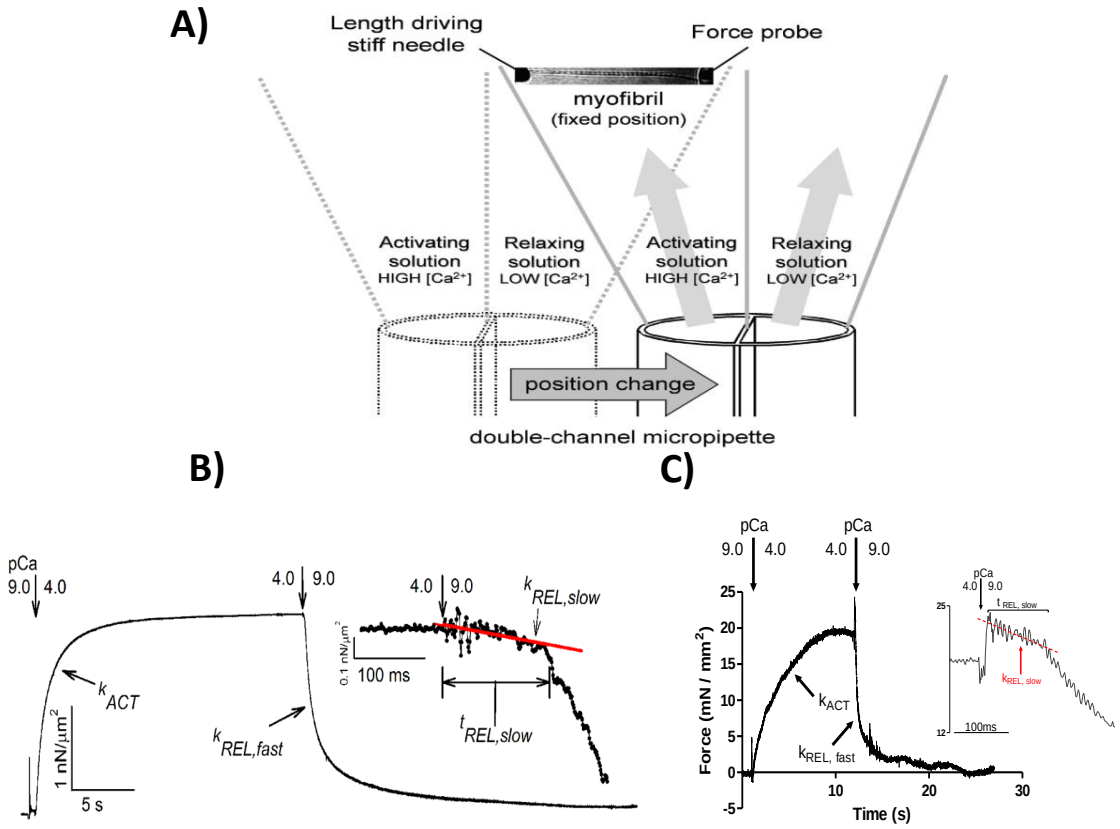

Figure 8. Measurement of human cardiac myofibril mechanics & kinetics.

A) Isolated myofibrils from human myectomy samples 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. B) Following sudden calcium activation, the myofibril develops force with a rate - k_{ACT} - that has been shown to reflect apparent cross bridge turn-over. 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 k_{REL} rates. C) A similar protocol for myofibril from iPSCs.

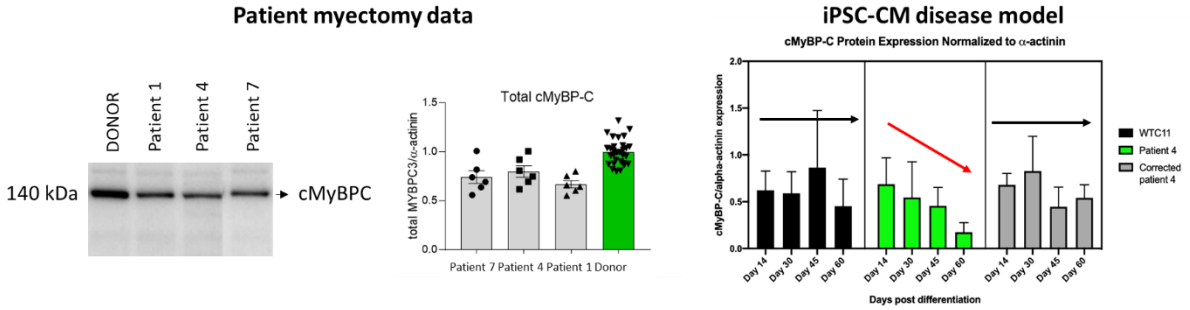

Figure 9. E258K mutation in *MYBPBC3* causes cMyBP-C haploinsufficiency in both the patient samples and in the iPSC-Cardiomyocyte *in vitro* model of the disease.

(Right) 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. (Middle) Expression of cMyBP-C normalized to α-actinin. cMyBP-C/α-actin in donor is set to 1 (donor N=1; E258K N=3). (Left) 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.

3.2 2'deoxy-ATP (dATP), a small molecule that enhances cardiac function in heart failure

This section reproduces a report on a collaboration between Task 2.1 and 2.2 of work done in parallel in with dATP in cardiac cells (2.1) animal hearts (2.2). See D2.2 Section 2.3.3.

The UW group previously demonstrated that elevating the level of dATP (a naturally occurring nucleotide) in failing myocardium, resulting from myocardial infarct, rescues cardiac function in rodent [19] and pig [8] models. Other previous studies (reviewed in [20, 21) provide a firm mechanistic basis, demonstrating that when cardiac myosin uses dATP in place of ATP there is an increased myosin activation and faster actin-myosin (crossbridge) cycling. In a just published report [22] 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 (P7,[23])) and how dATP effects on myosin-actin crossbridge cycling drives increased twitch contractions in heart muscle (P9).

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 modelling 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 (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.

4. Collaboration with WP5 MUSICO modelling

4.1 Cross bridge cycle data in solution and MUSICO modelling (Table 1, P2 & P4)

We have collected complete data sets for mutations in human cardiac myosin linked to HCM and DCM. This data is being used with the MUSICO software to analyse if the data can be used to predict HCM vs DCM phenotypes. Following on the publication of the data on DCM samples in 2018 [13] we have now analysed data on four myosin mutations linked to juvenile onset HCM and 3 linked to adult onset (P4).

In addition to the work on early and late onset HCM discussed above (P4) a further work was published utilising the MUSICO platform. In previous work we had characterised the motor domain of all seven major human muscle myosin isoforms and explored how the crossbridge cycle differed between the isoforms. In P2 (The ATPase cycle of human muscle myosin ii isoforms: adaptation of a single mechanochemical cycle for different physiological roles.) we used the MUSICO platform to model the crossbridge cycle for each isoform, and predict key mechanical properties of muscle fibres expressing each isoform. These included maximum shortening velocity, maximum relative force and the economy of ATP usage during shortening and during isomeric contraction. Where such properties have been measured experimentally they agreed with our predictions. This is an important validation of the MUSICO platform and allows the properties of muscles containing rare isoforms to be predicted, as well predicting the effects of myosin mutations on mechanical properties.

4.2 Multiscale Modelling of Twitch Contractions in Cardiac Trabeculae (P6 in press)

Sections 4.2 and 4.3 outline published work which is the outcome of a collaboration between Tasks 2.1, 2.2 and WP5. Detailed molecular and cellular level data collected by participants in Task 2.1 were combined with the internal force and calcium transient information collected by Task 2.2. The combination allowed the building of models within WP5 using MUSICO to generate transients that matched experimental tension transient. These sections of the report are reproduced in both D2.1 and D2.2 (Sections 2.6.2 and 2.6.3).

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 modelling has the potential to provide detailed insight into each of these processes, how the dynamics of each process affects the complexity of contractile behaviour 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 lengthening during relaxation.

Here we use the multiscale MUSICO platform to model rat trabecular twitches. 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 twitches in either an isometric sarcomere or in a fixed length, multiple sarcomere trabeculae replicate the experimental observations, 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 (Figure 10).

This multiscale approach is now being used to explore twitches in muscle fibers containing mutations associated with HCM & DCM – see next Section 4.3.

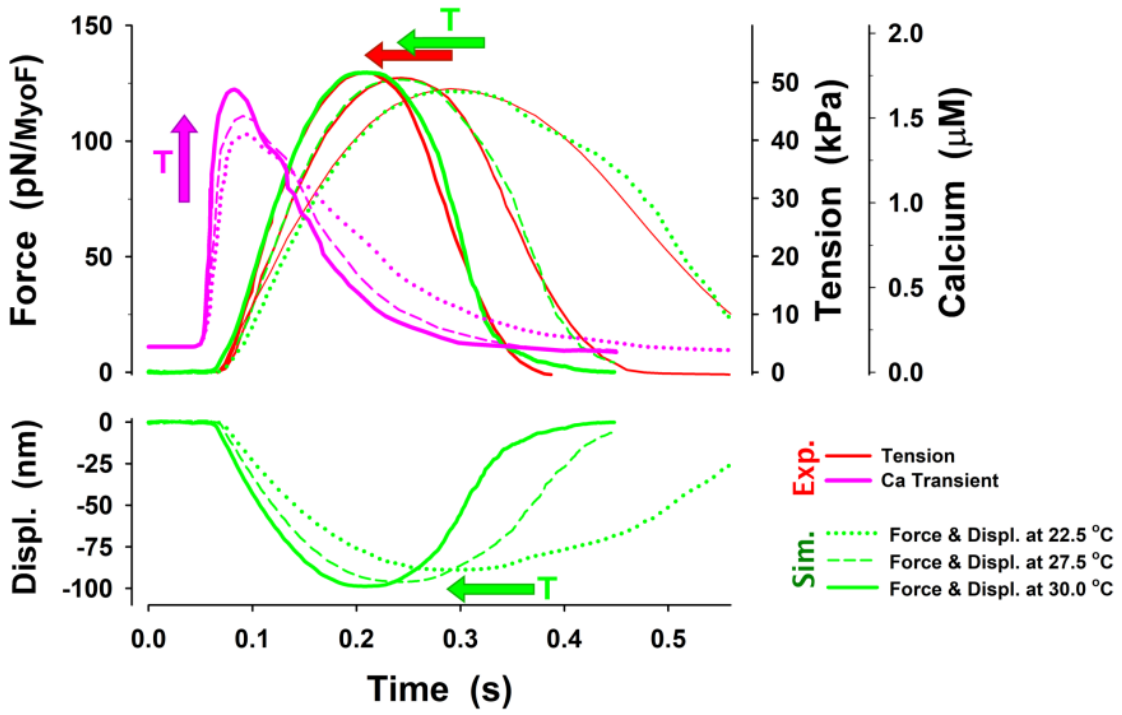

Figure 10. Modelling a rat trabeculae twitch contraction at three temperatures.

The MUSICO simulation with the six state crossbridge model (*green lines*) matched well the force responses (*red lines*) at 22.5 °C, 27.5 °C and 30 °C data from Janssen et al 2002 [24]. The rise of temperature increased peak calcium concentrations and relaxed faster (*pink lines*). The force transients followed the similar patterns having faster rise times, modest increase in peak force and a faster relaxation phase. The modelling required increase in crossbridge binding and ADP release rates with increasing temperature. Broad arrows indicate direction of change as temperature increased.

4.3 The Effect of Variable Troponin C Mutation Incorporation in Thin Filaments on Cardiac Muscle Twitch Contractions (Table 1, P8)

One of the complexities of understanding the pathology of familial forms of cardiac diseases 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 mutations of cardiac TnC, L48Q and I61Q, that decrease or increase the rate constant (k_{-Ca}) for the release of Ca^{2+} from cTnC resulting in HCM and DCM respectively. Expression of these mutations in transgenic mice results in incorporation of 30 and 50% respectively.

Here we demonstrate that fixed length 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 4.2 above), we were able

to describe the tension transients of WT mouse myocardium. Tension transients for the mutant TnCs were described with changes in k_{-Ca}, as measured in vitro for mutant TnC, a change in the affinity of TnC for TnI, and a reduction in the steady state level of detached crossbridges (Figure 11). 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 (Figure 12), but also the effect of variations in the way the mutant and WT TnC's are distributed throughout thin filaments in sarcomeres (Figure 13). 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.

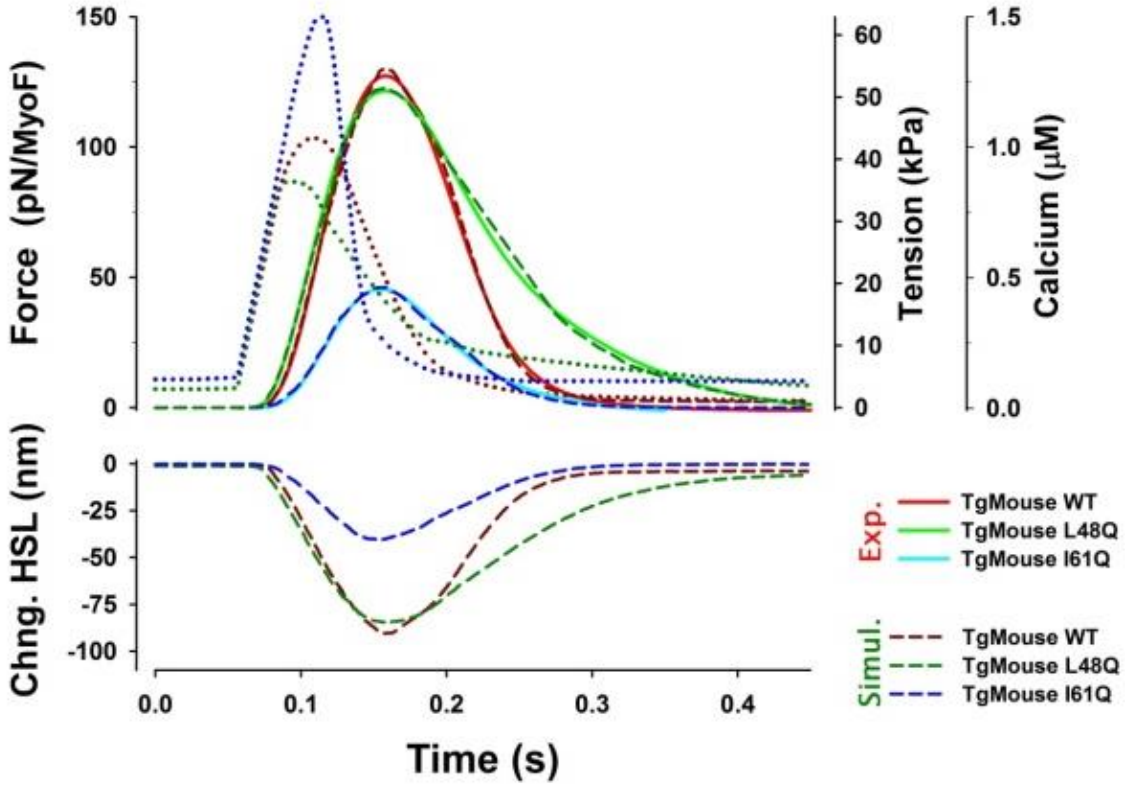

Figure 11. Modelling trabeculae twitches of transgenic mice for WT and two TnC mutations L48Q and I61Q.

Average observed values for twitch contractions (solid lines), compared to MUSICO predictions (dashed lines) of twitches for $cTnC^{WT}$(red), $cTnC^{I61Q}$ (blue) and $cTnC^{L48Q}$ (green) transgenic mice trabeculae at sarcomere length 2.3 µm. The twitches are driven by the Ca^{2+} transients (dotted lines) from Davis et al 2016 [25], and the model required the measured change in Ca^{2+} affinity for each TnC. Estimates of half sarcomere length changes (Chng. HSL).

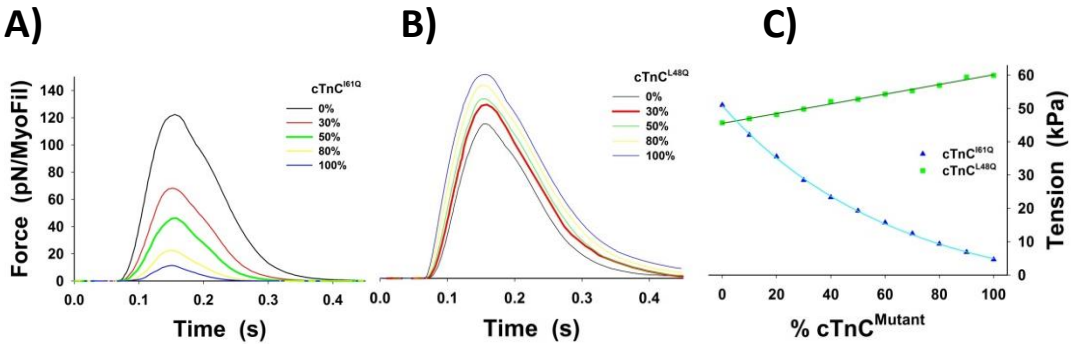

Figure 12. Modelling the effect of level of expression of TnC mutations in mouse trabeculae.

Predicted effect of % incorporation of cTnC^{I61Q} (A) and cTnC^{L48Q} (B) on twitches. C) Peak force as a function of % incorporation (Δ) cTnC^{I61Q}, (□) cTnC^{L48Q}.

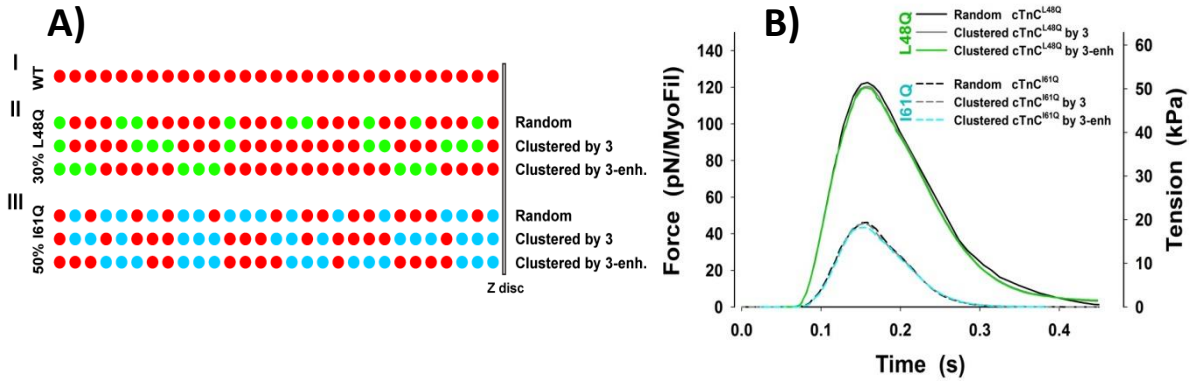

Figure 13. Modelling the effect of clustering of mutated TnCs mutations actin filaments.

A) Different random distributions of mutant TnC within a representative strands of actin filaments if mutant TnCs tend to cluster: I. cTnCWT (red dots), II. Random and clustered distributions of 30% cTnCL48Q (green), and III. Random and clustered distributions of 50% cTnCI61Q (blue). 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." B) 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). Clustering has little effect.

5. Deviation from the work plan

There have been three deviations from the work plan, all excepted through Amendments on GA.

1. The WP2 start date was pushed back because the grant contracts were not in place until quite late. Hiring of staff could not happen in time so the start date was pushed back by 3 months
2. At the kick-off meeting, in June 2018 it became apparent that there had been a significant change in focus of current research on HCM mutations in myosin and MyBP-C since the project proposal was submitted. This required a re-appraisal of our initial targets. A no-cost extension was agreed.
3. This is a laboratory based WP and as such was severely disrupted by the Covid 19 pandemic restrictions. Labs were closed for a period of 2-4 months in different countries. Therefore, both Tasks 2.1 and 2.2 were extended to M36.

There have been no other deviations from the work plan.

6. Conclusions

6.1 Summary of achievements

6.1.1 Several new methods developed to allow study of heart patient derived samples

a. *In vitro* models of FCMs have been created by differentiating hiPSCs to cardiomyocytes. Work started to validate the model has been successful.
b. UNIKENT has shown it is possible to purify myosin and myofibrils from both myectomy samples (UNIFI) and from iPSCs (UW).
c. To maximise the benefits of the small human samples available in (b) we have developed a micro volume stopped flow system using 25% of the material normally used and shown assays can be performed using a few µg of myosin or myofibrils.
d. We have devised a novel assay of the SRX state of myosin using myofibrils derived from heart tissue. This allows the assay of the SRX from both biopsy material and iPSCs.

6.1.2 The collaboration with WP5 has validated and extended the MUSICO modelling capability

a. The crossbridge cycle for all eight major sarcomeric myosin isoforms have been modelled and predictions for the mechanical behaviour of muscle cells expressing these isoforms agree with published data where available. These include the human α- & β-cardiac isoforms – currently little information is available on the mechanics of contraction of α-cardiac myosin which is predominant in the atria.
b. The contraction cycle has been modelled for 6 DCM linked mutations. All predict serious deficiency in the mechanical behaviour of cells carrying the mutation
c. A single contraction relaxation cycle (a twitch) provides a challenge for modelling and achieving validates our approach. Based on studies of isolated proteins and contractions of a few myocytes MUSICO successfully modelled the twitch of rat trabeculae collected by different labs and at different temperatures.
d. Using the same approach, the mouse cardiac twitch was modelled and the twitches of transgenic mice carrying mutations in TnC that either increase or decrease Ca affinity. These mutations mimic HCM and DCM phenotypes in the mouse. The twitches were successfully modelled using just the change in affinity measured in solution of purified proteins.

6.1.3 Analysis of FCMs

a. A set of mutations in human myosin were analysed associated with early and late onset of HCM. No major distinction was found between the crossbridge cycle of the two groups but both groups were distinct from myosin associated with DCM (Section 6.1.2b)
b. Patent samples (myectomy and iPSCs) carrying a unique mutation to the Tuscany region were analysed. The mutation is in MyBP-C and both myectomy and iPSCs indicated loss of MyBPC in myocytes compared to controls suggesting haploinsufficiency. Analysis of an

activation relaxation cycle in myofibrils indicated faster rate of tension rise, faster relaxation and increase tension cost of contraction.

6.2 Future plans

In addition to completing the publication of submitted work (Table 1, P8) there are several ongoing projects.

Novel in vitro assay of the SRX using patient derived samples (iPSCs and myectomy samples).

The model developed for the simulation of rat and mouse twitches will be adapted to simulate the data from human atrial and ventricle cardiac samples described in Section 4.2. Attention will be paid to the model predictions of conditions in which the relative expression of the fast and slow isoforms (α & β) of cardiac myosin is modified.

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.

Almost all HCM & DCM linked mutations are heterozygous meaning that both WT and mutant are expressed at variable levels in cells. This is very hard to address experimentally but can be addressed using the MUSICO modelling, as demonstrated for the TnC mutations in Section 4.3, provided the behaviour of both pure WT and mutant are known. We will collect such data to support the modelling effort.

Complete the experimental characterization of the E258K iPSC-cardiomyocyte model and start its use as a tool for drug testing.

Expand the iPSC-cardiomyocyte model to additional human HCM/FCM models.

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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