Final Report Summary - BIG-HEART (Bench-to-beside Integrated approach to familial hypertrophic cardiomyopathy: to the HEART of the disease)

Executive Summary:

Hypertrophic cardiomyopathy (HCM), characterised by left ventricular hypertrophy and myocyte disarray, is the most common cardiac single gene disorder. With a prevalence of 1:500, HCM is predicted to affect approximately one million people within the EU. HCM represents an important clinical problem, being the principal cause of sudden death in young adults, and a valuable opportunity to use human genetics to dissect mechanisms of cardiac hypertrophy and heart failure. It was the first inherited heart disease to be characterised at the molecular genetic level, with the demonstration that it is caused by mutations in genes that encode different components of the cardiac contractile apparatus.

The BIG-Heart (Bench-to-bedside InteGrated approach to familial hypertrophic cardiomyopathy: to the HEART of the disease) consortium was assembled in order to increase understanding of disease mechanisms in HCM and to use this knowledge to identify tractable therapeutic targets. Existing studies had indicated that HCM mutations enhance contractility and impair relaxation; we hypothesised that these changes in cardiac contractility may result in energetic compromise, due to inefficient ATP utilisation, and also in altered intracellular Ca2+ handling. Most of previous work had focused on in vitro experiments and mouse models and not on characterising human cardiac muscle from HCM patients. Hence one of the principal aims of the project was to collect and genotype a large collection of both affected HCM and control human myocardium and to measure the differences in protein expression, contractility, protein phosphorylation and sarcomeric structure between normal and affected samples. Furthermore we set out to test therapeutic strategies in existing mouse models and to employ patient-based studies to test specific interventions based on existing hypotheses. This provided a broad multidisciplinary approach to gain further understanding of HCM and to yield new directions for therapeutic strategies.

BIG-Heart has achieved its principal aims and has made significant advances towards greater understanding of HCM. Furthermore it has succeeded in disseminating its progress to the scientific community and to the wider audience of patient groups and the public. We acquired human heart tissue from HCM patients and control donors throughout the project, and amassed a total of 226 HCM samples and 111 controls. This has proved a valuable and unique resource for the characterisation of the changes in HCM human myocardium. We have shown that higher myofilament Ca2+-sensitivity is partly due to reduced phosphorylation by protein kinase A. Length-dependent activation has been found to be significantly lower and the level of troponin I phosphorylation is uncoupled from myofibrillar Ca2+-sensitivity. Consistent with energetic compromise in HCM, we have demonstrated excess tension cost and reduced efficiency in HCM compared with control sarcomeres. Animal models, incorporating the genetic mutations found in HCM patients, have been generated. These allow determination of HCM cardiac phenotypes in vivo; treatment of HCM mouse models (e.g. with perhexiline and ranolazine) has gained insights into drug mode of action and has allowed us to evaluate their potential for clinical trials. Electrical abnormalities have been identified in cardiac myocytes from HCM tissue and mouse models that underlie the risk of cardiac arrhythmias. Spliceosome-mediated RNA trans-splicing (SmaRT), a new tool for RNA-based therapy of genetic diseases, has
successfully been employed to “repair” a HCM-causing MYBPC3 mutation in cells and a mouse model. Importantly, this approach has improved cardiac function in the animal model, hence is a promising concept which could be explored for application in humans. Proof-of-concept has also been demonstrated for a second RNA-based strategy involving exon skipping. Among the clinical advances, the BIG-Heart HCM score has been developed; this provides a numerical assessment of risk stratification based upon known parameters and gives the prospect of tailoring clinical resources for HCM patients more precisely.

Project Context and Objectives:

Cardiovascular disease in the European Union (EU) is responsible for over 1.5 million deaths annually and is the chief cause of sickness and morbidity of its citizens. The associated healthcare costs are immense; the associated costs of cardiovascular disease in the EU costs are estimated to be at least €169 billion annually. There are both acquired and genetic bases of cardiovascular disorders, with most disease being multifactorial and resulting from the interaction of numerous environmental and genetic factors. However there are a number of single gene disorders of the cardiovascular system, inherited in a simple Mendelian fashion. The most prevalent of these is the autosomal dominant disease hypertrophic cardiomyopathy (HCM) which has a reported frequency of 1:500 and thus is likely to be affect approximately one million people within the EU.

HCM is characterised by hypertrophy of the left ventricle in the absence of other cardiac or systemic disease (such as hypertension or aortic stenosis). There is hypertrophy of both the left ventricular wall and intraventricular septum, resulting in a decreased left ventricular volume. Obstruction of the left ventricular outflow tract is present at rest in approximately 25% of patients and in a higher proportion during exercise. Histologically, areas of myocyte disarray and interstitial fibrosis are characteristic of the disease. Although systolic contractility is preserved and features of hypercontractility are observed at the whole heart level, outflow obstruction and impaired relaxation can cause progressive forward and backward heart failure and an increased incidence of ventricular arrhythmia can lead to sudden cardiac death. Molecular genetic work has shown that HCM is caused by mutations in genes that encode components of the contractile apparatus of the heart, leading to it being labelled a disease of the sarcomere. In vitro and in vivo studies have suggested that the primary effect of HCM mutations is to enhance contractility and impair relaxation. These changes in contractility are predicted to result in energetic compromise, due to inefficient energy utilisation, and also to cause alterations in calcium handling, protein phosphorylation and gene expression.

The BIG-Heart (Bench-to-bedside InteGrated approach to familial hypertrophic cardiomyopathy: to the HEART of the disease) consortium was assembled in order to test specific hypotheses that have emerged from recent reductionist studies of disease mechanisms in HCM. If validated in the human heart, these hypotheses would identify tractable therapeutic targets that suggest that HCM, perhaps more than any other cardiomyopathy, will be amenable to disease modifying therapy.

The principal objectives of BIG-Heart have been:

- To establish and genotype a substantial collection of affected human HCM myocardium and to collect, for the first time, a set of appropriate control myocardial samples;
- To systematically measure the differences in contractile parameters, protein expression and phosphorylation, electrophysiology and sarcomeric structure between normal and affected samples;
- To evaluate the contribution of perturbations of energetics and calcium handling as central components leading to hypertrophy, contractile dysfunction and arrhythmia;
- To test therapeutic strategies in our existing mouse models;
- To employ patient-based studies to evaluate aspects of disease progression and to test specific interventions based on current hypotheses.

The BIG-Heart project has brought together five scientific groups, each with leading expertise in the analysis of human heart
muscle, the creation of mouse models of cardiomyopathy, and in patient-based studies; hence the planned experiments have built on the existing strengths of the individual laboratories. Prior to the BIG-Heart project start, there was pairwise interaction between some of the partners; this has now been considerably enhanced through the open sharing of material and data are leading to synergy in our work towards our common goal.

As well as the clear potential impact on the specific scientific field of the study of HCM, BIG-Heart also aimed to impact on wider aspects of cardiovascular and other medical science through the development of novel techniques that would have broader applications. Among these, the project aimed to develop protocols for the exchange of troponin-tropomyosin in human cardiac myofibrils and use of phosphate affinity electrophoresis to identify phosphorylated forms of various cardiac contractile proteins. The testing of mRNA-based “gene therapy” strategies such as Spliceosome-mediated RNA trans-splicing (SmaRT) was an important component of BIG-Heart; the successful use of this approach would represent the first demonstration of this strategy in heart muscle with obvious application of the technique for other cardiac disorders.

The consortium aimed to disseminate the advancement of knowledge within the BIG-Heart, to the wider scientific community and to the general public including patient groups. It was planned to disseminate within the consortium via regular meetings, newsletters/emails and conference calls to inform partners of the progress of the Science and Technology activities. A website was proposed to aid with all aspects of dissemination; the private section of website was to be designed as a tool to communicate and disseminate meeting presentations, methods, protocols and deliverables internally. Engagement with the scientific community was planned to take place at local, national, European and international levels, and the publication of results in scientific journals and the participation in conferences and symposia were designed to be key components of this. A dissemination strategy to raise awareness of the general public of BIG-Heart’s activities and to communicate the progress in the advancement of knowledge was planned. The website was expected to be central to this, acting as a portal for HCM patients to gain access to the information.

The BIG-Heart work has been divided into Work Packages (WPs) to allow efficient administration and organisation of this large project. There have been seven WPs devoted to experimental work (WPs 1 to 7) plus one each for dissemination and management (WP8 and 9 respectively). The themes of experimental WPs were:

WP1 – Collection and genotyping of HCM and control Human Tissue
WP2 – Determination of the contractile parameters of normal and affected myocardium
WP3 – Quantitative analysis of contractile proteins in normal and affected human myocardium
WP4 – Determination of the electrophysiological properties of isolated myocytes
WP5 – Determination of the alterations to sarcomere structure caused by MYBPC3 mutations
WP6 – Interventional approaches in models of HCM
WP7 – Clinical investigations
WP8 – Dissemination
WP9 - Management

Project Results:

WP1 – COLLECTION AND GENOTYPING OF HCM AND CONTROL HUMAN TISSUE

Start month: 1
End Month: 42
WP Leader: CO1 UOXF.HS
Task 1.1 Collection of human myocardium samples

A central theme of the BIG-Heart project has been the collection of human myocardial samples from both HCM patients and controls in order to make a comprehensive analysis of the differences in contractility and muscle structure that characterise HCM. Cardiac tissue from HCM patients has generally been obtained from septal myectomy procedures. Normal control myocardium has been obtained from patients with mitral stenosis or atrial septal defect and normal left ventricular function. Samples from patients with cardiac hypertrophy not caused by a primary genetic defect were also obtained, for example, during surgery for aortic stenosis, or cardiac catheterization of patients with hypertensive heart disease. Collaborations were established with relevant clinical centres including the Erasmus Medical Center in Rotterdam (P2 VUMC) and via the biobank programme of P3 UniFI. At the conclusion of BIG-Heart, our collection comprised 226 HCM samples along with tissue from 33 normal controls and 78 patients with acquired (secondary) left ventricular hypertrophy, in each case exceeding the number of samples predicted at the start of the project. This unique collection has formed an important central resource for BIG-Heart, available to all partners and has been extensively used in the series of structural, functional and proteomic investigations described in WPs 2-5.

Task 1.2 Identification of HCM-causing mutations in patients

Screening for mutations in at least the eight most common HCM genes (MYH7, MYPBC3, ACTC1, TNNT2, TNNI3, TPM1, MYL2, MYL3) has taken place in over 70% of the samples in the collection. In common with large cohort studies [1], disease-causing mutations were found in approximately two thirds of the samples screened. As expected, the most common disease genes were found to be MYBPC3, with mutations detected in 29% of samples, and MYH7, in 12% of samples. Ten mutations in thin filament proteins (encoded by TNNT2, TNNI3, TPM1 and ACTC1) were detected; despite these being relatively rare causes of HCM, they are of particular mechanistic interest and were extensively studied in WPs 2 and 3.

Task 1.3 Coordination of study on human samples

Prior to collection of the samples, it was ensured that the appropriate ethical approval was obtained by each centre and copies of these documents were sent to CO1 OXF.HS for collation. Standardised protocols were developed to ensure reproducible sample preservation independent of the collecting centre including an alternative protocol for fixation of tissue for electron microscopy (required for the work described in WP5). A Filemaker Pro database was established, holding information about the collected human samples: sample characteristics, patient information (genetic and clinical data) and results of experiments. The database, currently with 302 entries, is available online, via a secure website and access is limited to BIG-Heart partners. The database allows online modification and addition of datasets, allowing project partners to update several experimental results on the same sample.

Deliverables

. D1.1 Description of a database of HCM and control human myocardium samples, detailing disease-causing mutation, clinical description, amount and location of material and functional characteristics (month 6).
. D1.2 Collection of copies of Ethical approvals (month 6).

WP2 Determination of contractile parameters of normal and affected myocardium

Start month: 1
End Month: 42

WP Leader: P3 UniFI
Task 2.1 Measurement of myofilament Ca2+-sensitivity in human myofibrils, myocytes and in vitro motility

Using the HCM tissue collection of the BIG-Heart consortium (see WP1), we were able to demonstrate that high myofilament Ca2+-sensitivity is a common characteristic of human HCM independent of the exact mutation and sarcomeric protein involved. This feature of HCM can contribute to arrhythmogenesis and diastolic dysfunction (see also WP4). A list of potential targets and tools to decrease sarcomere Ca2+-sensitivity in HCM has been reported (Deliverable 2.3).

The high myofilament Ca2+-sensitivity found in human HCM samples partly reflects hypo-phosphorylation of PKA-targets compared to non-failing donors [2]. In vitro function of TnT isolated from human HCM samples is abnormal irrespective of the mutation causing the disease [3]. The abnormal TnT function seems responsible for uncoupling TnI phosphorylation from changes in myofibrillar Ca2+-sensitivity. This uncoupling may play a role in the pathogenesis of HCM.

Length-dependent activation is significantly smaller in all HCM samples vs. donors. The effect may be due to different mechanisms that depend on specific mutations but seems to represent a common patho-mechanism in HCM [4]. The mutation-induced impaired length-dependent activation may limit the preload-mediated contractile reserve of the heart.

A technique to extract and replace Troponin (Tn) and Tropomyosin (Tm) into isolated myofibrils has been developed (Deliverable 2.1; [5]). The technique has been used: (i) to compare the impact of Tm HCM mutations in homodimeric and heterodimeric forms on myofilament Ca2+-sensitivity and other biophysical properties of the contractile apparatus [6]. The results indicate that the study of the effects of the homodimeric forms of Tm is not necessarily a good predictor of the functional impact of Tm HCM mutations; (ii) to investigate the biophysical and functional impact of Tm phosphorylation by using pseudo-phosphorylated and phosphorylation-null engineered Tms [7]. The results suggest that Tm phosphorylation slows thin filament deactivation and plays a role in the regulation of relaxation dynamics.

Task 2.2 Human cardiac sarcomere relaxation measured in myofibrils

Faster rates of early isometric tension decay following Ca2+ removal that reflect faster apparent rates of cross bridge dissociation are a common feature of human HCM myofibrils carrying both MYH7 and thin filament regulatory protein mutations compared to control groups [8]. Nonetheless, overall relaxation that is shorter and faster in MYH7 mutant myofibrils, is often prolonged and incomplete in thin filament mutant myofibrils suggesting impairment of the mechanisms that switch contraction off in the latter HCM group. This indicates that potential mutation-related differences in the patho-mechanisms may lead to HCM diastolic dysfunction. Diastolic dysfunction in MYH7-mutant HCM hearts is likely to be related to secondary changes in energetics and E-C coupling of HCM myocytes (see Task 2.4 and WP4) rather than to primary effects of myosin mutations. Overall relaxation impairment observed in myofibrils from thin filament mutant HCM patients provides additional mechanisms that primarily relate protein mutations with diastolic dysfunction. The idea that sarcomeric mechanisms may reinforce E-C Coupling (ECC) abnormalities responsible for diastolic dysfunction in thin filament mutant hearts is strengthened by the finding that, at variance with thick filament mutant myofibrils, resting tension is higher in thin filament mutant myofibrils compared to control myofibrils. Although our findings are based on a relatively small number of mutations from different genes, and hence of preliminary nature, the results may be useful to drive future work towards potential mutation-specific therapies.

Task 2.3 Force generation and cross bridge kinetics in human cardiac sarcomeres determined in myofibril and myocytes and in vitro motility

Human HCM is usually associated with some impairment of the sarcomere maximal tension generating ability [9]; in most cases this can be related to reduced myofibril density and secondary hypertrophy remodelling. Specific impairment of the force generating ability of the human cardiac HCM sarcomere is associated with mutations in specific genes (especially MYH7). Work, still in progress, suggests that impairment of maximal tension is more severe in preparations from HCM patients with
complex genotype.

Several HCM mutations in sarcomeric proteins are associated with faster apparent cross bridge turnover measured in isolated myofibrils. Apparent cross-bridge kinetics, instead, are not different in myofilament-mutation-negative HCM myofibrils compared to controls. Exchange experiments have provided evidence that some mutant proteins (e.g. TNNT2 K280N) are directly responsible for the kinetic changes.

The faster cross bridge turnover associated to HCM mutations is mostly due to increased isometric cross bridge detachment rate that may lead to inefficient energy utilization by the sarcomere (see also Tasks 2.2 and 2.4).

Task 2.4 Measurement of the tension cost in demembraned multicellular preparations.

To test the energetic compromise hypothesis of HCM, isometric ATPase activity was measured simultaneously with force in a large number of skinned multicellular trabeculae from HCM patients carrying different myofilament mutations, sarcomere-mutation-negative HCM patients, aortic stenosis patients and donor samples. Analysis of results is still in progress but clearly significant results have been obtained. Specifically:

(I) Tension cost in myofilament-mutation-negative HCM preparations is not significantly different from that measured in aortic stenosis samples, in agreement with myofibril measurements of apparent cross bridge kinetics (Task 2.3);
(II) Excess ATP utilization in HCM patients carrying the MYH7 R403Q mutation and in the homozygous TNNT2 K280N mutant patient, previously suggested by kinetic measurements of cross bridge detachment rate under isometric conditions (Tasks 2.2 and 2.3) has been confirmed by the direct measurement of tension cost; interestingly, in the case of the TNNT2 mutation, replacement of the mutant protein with wild type (WT) recombinant cTnT reduced the tension cost towards control levels.

Additional work on tension cost in HCM has been performed in mouse models of HCM. Skinned trabeculae from a novel cTnT E163R HCM mouse compared with WT trabeculae exhibited a significant increase (about 50%) of tension cost, compared with WT mice. Measurement of the energy (work and heat) released during twitches of intact heart muscle preparations from ACTC1 E99K HCM and non-transgenic (NTG) mice showed that the work cost in terms of energy turnover was disproportionately higher in the ACTC1 E99K mutant mice than in NTG mice. Recovery energy was similar for ACTC1 E99K and NTG indicating that the altered efficiency was predominantly cross-bridge based. The changes in the direct measurement of efficiency in intact HCM preparations correlate with the measurements of tension cost described here by direct measurement of ATPase and tension in skinned preparations [10].

Deliverables

D2.1 Novel protocol for troponin-tropomyosin extraction and reconstruction in human cardiac myofibrils (month 18).
D2.2 List of inventions effective in reducing Ca2+-sensitivity of HCM myofibril and myocyte preparations (month 39).
D2.3 Report on the development of a novel exchange protocol in human cardiomyocytes to restore cMyBP-C in MYBPC3 mutation induced haploinsufficiency (month 36).

WP3 Quantitative analysis of contractile proteins in normal and affected human myocardium

Start month: 1
End Month: 42
WP Leader: P2 VUMC
Task 3.1 Expression of mutated protein in human heart

Our previous studies [11, 12] did not show any evidence of truncated cMyBP-C protein in HCM patients harboring mutations in MYBPC3. The absence of any detectable truncated cMyBP-C argues against its incorporation into the myofiber and any dominant negative effects. The lowered relative level of full length protein in both truncation and missense MYBPC3 mutations argues that haploinsufficiency is sufficient to cause the disease [13]. Our recent comparison of HCM samples with truncating mutations in MYBPC3 (MYBPC3mut) and sarcomere mutation negative HCM samples (HCMsmn) showed that the lower level of full length protein was specific for the MYBPC3mut group. The variation in protein expression is rather large, and several samples showed cMyBP-C expression levels close to normal. This suggests that haplo-insufficiency is not the sole determinant of disease onset in HCM. Changes in post-translational modifications may contribute to disease development, of which phosphorylation was most extensively studied during the BIG-Heart project. Within our future studies we aim to combine changes in expression and post-translational modifications in order to understand their combined effects on cardiac function.

P4 UKE has evaluated the consequence of human HCM mutations in MYBPC3 at the mRNA level. Samples were analysed from P2 VUMC, P3 UniFl and P5 Imperial. For the first time using human cardiomyopathy samples, the study demonstrated that two different mutations located on the last nucleotide of exons, which is part of the consensus splice site sequences, result in skipping of the corresponding exons and explains how some missense mutations in cMyBP-C can cause premature chain termination leading to haplo-insufficiency. This study underlines the need i) to systematically analyse the consequence of the mutation at the mRNA level, and ii) to give the cDNA name of the mutation rather than the protein name, which may be confusing.

In addition to MYBPC3mut samples, we have investigated a unique HCM sample harbouring a homozygous TNNT2 mutation. Genotyping indicated a novel potentially disease-causing homozygous missense mutation c.804G&gt;T in the TNNT2 gene predicted to produce a K280N mutation in cardiac troponin T. The homozygous mutation was confirmed at the cDNA level and at the protein level by mass spectrometry, where the TNNT2 K280N variant was detected and the wild-type protein was absent. In 1D-gels, the protein content of cTnT relative to actin was indistinguishable from wild-type indicating that there was no haplo-insufficiency and the mutation could act as a poison peptide. Mass spectrometry also showed that the isoform of cTnT expressed was TnT3 in both donor and mutant heart samples.

Following on from our studies of troponin I, troponin T [3] and actin [14] isoform changes in HCM we also investigated expression of tropomyosin isoforms, finding that in human heart &gt;95% of tropomyosin was the striated muscle alpha isoform (alpha tropomyosin isoform 1, P09493) and this did not change in HCM or heart failure [15].

Task 3.2 Phosphorylation of contractile proteins in human heart

Post-translation modifications of sarcomeric proteins, in particular phosphorylation, are important regulators of myocardial properties. In our functional studies we observed high myofilament Ca2+-sensitivity and a blunted length-dependent increase in Ca2+-sensitivity in all HCM samples compared to non-failing donor myocardium. To determine alterations in protein phosphorylation in diseased myocardium from HCM patients we have developed novel sensitive methods to carefully dissect phosphorylation patterns. Phospho-specific antibodies and ProQ Diamond staining were used to study changes in cMyBP-C phosphorylation patterns. In addition, Phos-Tag analysis was developed and published to study different phosphorylation species of cTnI, cMyBP-C and MLC2 [16, 17, 18]. In our most recent study [4], 30 patients carrying a heterozygous mutation in MYBPC3 (n=21; MYBPC3mut), MYH7 (n=6; MYH7mut), TNNI3 (n=2; TNNI3mut) and TPM1 (n=1; TPM1mut), were studied. The MYBPC3mut group consisted of patients with truncating (n=17) and missense (n=4) mutations. Phosphorylation of cMyBP-C and cTnI were significantly lower in all patient groups, although as found by van Dijk et al. [2], cMyBP-C phosphorylation corrected to cMyBP-C expression level is not different in HCM with MYBPC3 mutations compared to donor. In addition, phosphorylation in an explanted heart with a homozygous TNNT2 K280N mutation phosphorylation levels were relatively high. However, overall PKA-mediated phosphorylation is lower in HCM compared to non-failing hearts. A similar observation has
been made in the Mybpc3 knock-in (KI) model in which troponin I phosphorylation was significantly lower than control. Low phosphorylation may be due to reduced PKA activity or increased phosphatase activity, but could also be explained by mutant-induced changes in the protein substrate. Therefore, we investigated whether treatment of HCM samples with exogenous PKA would still be able to increase phosphorylation levels to those found in non-failing heart samples.

Analysis of phosphorylation showed that cMyBP-C phosphorylation after PKA was close to values observed in donor samples in all HCM samples. Analysis of phosphorylation at specific PKA sites (Ser275 and Ser284) on cMyBP-C confirmed increased PKA-mediated phosphorylation in HCM samples. Exogenous PKA also increased phosphorylation of cTnI in all HCM samples, although values did not always reach the level found in donor myocardium.

Based on these phosphorylation studies we can conclude that high myofilament Ca2+-sensitivity and perturbed length-dependent activation in HCM compared to donor are at least partly explained by secondary disease-related impairment of PKA-mediated phosphorylation. In WP2 we described how some of these effects may also result from uncoupling of the relationship between Ca2+-sensitivity and troponin I phosphorylation.

P3 UniFl has found many alterations in the kinetics of myofibril contraction and relaxation. These changes seem to be age-dependent. Until now, we do not exactly understand the basis of the changes in kinetics. Interestingly, we identified a novel phosphorylation site (Serine 133) in the linker domain of cMyBP-C, which may be involved in actin-myosin interactions. We have shown that Ser133 is target of the ‘hypertrophy’ kinase, glycogen synthase kinase 3β (GSK3β). GSK3β increased the rate of force redevelopment in human cardiomyocytes [19]. We will investigate post-project whether Ser133 phosphorylation is altered in HCM and whether this underlies altered kinetics in disease.

Deliverables

D3.1 Report on the use of the novel phosphate affinity SDS-PAGE technique (task 3.2) to quantitate the phosphorylation of cTnI, cMyBP-C and MLC-2 in human myocardium (month 12).

WP4 Determination of the electrophysiological properties of isolated myocytes

Start month: 1
End Month: 42
WP Leader: P3 UniFl

Task 4.1 Electrophysiological abnormalities in isolated human myocytes

A technique that allows separation of single viable myocytes from small samples of human ventricular myocardium has been developed. One main advance over previous techniques is provided by a custom made digestion device which delivers gentle mechanical stirring of tissue chunks, allowing single cell separation without excessive damage (see Deliverable 4.1 and [20]).

The electromechanical profile of cardiomyocytes from HCM patients undergoing myectomy was assessed and compared with that of cardiomyocytes from non-hypertrophic non-failing surgical patients by performing patch-clamp and intracellular Ca2+ studies. Compared with controls HCM cardiomyocytes showed prolonged action potential related to increased late Na+ (INaL) and Ca2+ (ICaL) currents and decreased repolarizing K+ currents, increased occurrence of cellular arrhythmias, prolonged Ca2+-transients, and higher diastolic Ca2+. Such changes were related to enhanced Ca2+/calmodulin kinase II (CaMKII) activity and increased phosphorylation of its targets. Ranolazine at therapeutic concentrations partially reversed the HCM-related cellular abnormalities via INaL inhibition, with negligible effects in controls. By shortening the action potential duration
in HCM cardiomyocytes, ranolazine reduced the occurrence of early and delayed after-depolarisations. Finally, as a result of the faster kinetics of Ca2+-transients and the lower diastolic Ca2+, ranolazine accelerated the contraction-relaxation cycle of HCM trabeculae, ameliorating diastolic function [21]. In agreement with an increase in CaMKII activity in HCM, acute administration of a CaMKII inhibitor, Autocamtide 2-related inhibitory peptide II (AIP II), significantly reduced diastolic intracellular Ca2+ levels of HCM cardiomyocytes. Consistently, diastolic tension was lower in AIPII-treated HCM trabeculae, and the increase of diastolic tension at high stimulation frequency was reduced. AIPII also improved contraction kinetics of HCM trabeculae, by shortening contraction peak time.

As conclusion, a specific set of functional changes in human HCM myocardium stem from a complex remodelling process involving alterations of CaMKII-dependent signalling, rather than being a direct consequence of the causal sarcomeric mutations. Among the several ion channel and Ca2+-handling proteins changes identified, an enhanced INaL seems to be a major contributor to the electrophysiological and Ca2+dynamic abnormalities of ventricular myocytes and trabeculae from patients with HCM, suggesting potential therapeutic implications of INaL and CaMKII inhibition.

Additional mechanisms underlying HCM cardiomyocyte adverse remodelling are under investigation. Among them there is a reduction of t-tubular density and function, documented by novel optical techniques [22, 23] and likely contributing to abnormal Ca2+-transient and twitch amplitudes and kinetics. The potential role of Ca2+-desensitizers in rescuing abnormalities related to t-tubule loss will be investigated post-project.

Task 4.2 Electrophysiological abnormalities in the ACTC1 E99K transgenic mouse model of HCM

The ACTC1 E99K transgenic (TG) mouse reproduces aspects of HCM seen in humans including sudden cardiac death (SCD) and apical hypertrophy. The ACTC1 E99K model is a particularly useful model to investigate altered electrophysiology and Ca2+-handling since this strain of mice are uniquely prone to SCD at early age (48% of females and 22% of males die between 28 and 45 days old), whilst survivors develop overt hypertrophy leading to dilated cardiomyopathy at 9 months. One main effect of this mutation is increased myofilament Ca2+-sensitivity [24]. We hypothesised that this increased sensitivity will disturb intracellular Ca2+-homeostasis, alter the function of key regulatory proteins and cause aberrant electrophysiology.

Electrocardiograms (ECGs) were compared between ACTC1 E99K and NTG mice at rest and under isoprenaline challenge. ECG abnormalities were observed in ACTC1 E99K mice, these were found to be age and sex dependent. Ventricular myocytes from ACTC1 E99K TG and non-transgenic (NTG) mice at 2 time points, 25-45 days (young) and 58-85 days (old), were isolated and used for a comprehensive investigation of excitation-contraction coupling features. Our studies have shown that the Ca2+-transient size and rate is enhanced in ACTC1 E99K TG in the SCD window which would be anti-compensatory whilst older surviving mice have a reduced Ca2+-transient. Mutation dependent changes in the sodium-calcium exchanger (NCX) are more exaggerated in female than male.

Task 4.3 Use of mouse models to develop treatments for SCD in HCM

To better investigate the potential benefits of ranolazine supported by the results on human HCM intact cardiomyocytes and trabeculae (Task 4.1) we extended electro-physiological, excitation-contraction coupling, and mechanical measurements to intact cardiomyocytes and trabeculae of HCM mice bearing the ACTC1 E99K mutation (Task 4.2) and a number of cTnT mutations. Acute in vitro and long term in vivo studies of the effects of ranolazine have been started in three cTnT mutant HCM mouse lines (R92Q, ∆160E, and E163R) generated from progenitors provided by Dr Jil Tardiff (University of Arizona, Tucson). In vitro, spontaneous Ca2+-waves and Ca2+-transients occur more frequently in the TnT mutant myocytes than in WT both at baseline and in the presence of β-adrenergic stimulation. Ranolazine significantly reduces the occurrence of these spontaneous events. In addition, in the mutant myocytes, ranolazine (i) lowers the amplitude of Ca2+-transients (ii) significantly reduces diastolic intracellular Ca2+, and (iii) accelerates Ca2+-transient kinetics. Taken together these results indicate that ranolazine could have both anti-arrhythmogenic potential as well as the capability to ameliorate diastolic properties (work still in progress).
Epigallocatechin-3-gallate (EGCG), predicted to be a Ca2+-desensitizer, was shown to decrease Ca2+-sensitivity of mouse cardiac myofibrils and human heart myofilaments in the in vitro motility assay. Its potential for reversing the enhanced Ca2+-sensitivity due to HCM mutations is under investigation.

 Deliverables

D4.1 Report on the optimized method for the isolation and handling of cardiomyocytes from fresh septal myectomy tissue to enable complete electrophysiological characterization (month 12).

WP5 Determination of alterations to sarcomere structure caused by MYBPC3

Start month: 1
End Month: 42
WP Leader: P5 Imperial

Task 5.1 Location and quantification of MyBP-C in thick filaments

We aimed to initiate studies on the sarcomeric structure of normal and diseased human heart muscle using our state of the art electron microscopy techniques. We established methodology for 3D visualisation of the arrangements of contractile proteins, intercalated disk and collagen in heart muscle by 2-photon confocal microscopy using antibodies to MyBP-C, α-actinin and cadherin, and second harmonic generation microscopy in unstained tissue. We demonstrated myocyte disarray in the ACTC1 E99K mouse model and developed a Fourier transform-based method for quantifying disarray.

EM images from human donor and HCM muscle were successfully obtained, and cMyBP-C was located in the myofibrils. We have established an image averaging protocol for analysis of the 1-D profile of the A-band of the sarcomere from electron micrographs. Initial studies showed a non-uniform distribution of the protein on the thick filament. These findings support haplo-insufficiency as potential disease mechanism for HCM. Due to the difficulty of obtaining normal human tissue, we compared the distribution to the A-band structure in normal frog skeletal, rat cardiac muscle and cardiac muscle of MyBP-C-deficient mice with MyBP-C mutations. Very similar overall profile averages were obtained from the C-zones suggesting that mutations in MyBP-C do not alter its mean axial distribution along the thick filament as shown by the prominent “forbidden meridionals” at 1/21.5 and 1/43 nm seen on the Fourier transforms. The MyBP-C bands are however less prominent relative to the M-band in the mutated samples compatible with haplo-insufficiency of cMyBP-C ([25];)

Task 5.2 High resolution structure of thick filaments with cMyBP-C mutations

New methodology was developed to allow high resolution tomography of human heart muscle samples. Previously, we analysed the structure of the thick filament using longitudinal sections; however this approach requires cryo-fixation and this is not possible for the human myectomy samples due to their haphazard availability. Therefore we developed methodology for transverse section tomograms. We demonstrated the principle of using thick transverse sections of muscle to rapidly construct tomograms of the arrangement of cMyBP-C on the thick filament. The technique has been applied to heart muscle from several transgenic mouse models with knockout or modified cMyBP-C. C-zones were selected carefully from transverse serial sections. We used sub-tomographic averaging to produce mean structures of segments of thick filaments in the C-zone. Initial results show a structure very similar to the one we previously obtained using longitudinal sections of frog skeletal muscle [26].

We also collaborated with Prof C. Gurnett on a study of MYBPC1 in zebrafish as a model of distal arthrogryposis [27]. In this
study the effect of mybpc1 depletion by mybpc1 antisense morpholino methods and the effects of overexpressing two human MYBPC1 mutations, W236R and Y856H, in the zebrafish mybpc1 gene were investigated. We carried out the structural analysis which showed great reduction of myofibril content and increased myofibrillar disorder in the mybpc1 morphant which caused lethality at 5 days. Overexpression of either missense mutation had negligible effect on myofibril structure.

Task 5.3 Structural analysis of Z-line architecture

The width of Z-line in the HCM MYBPC3 E542Q (MH1) sample was examined to analyse whether it differed with that of human myectomy control. The width is determined by the number of layers of alpha-actinin crosslinking the antiparallel actin filaments. Variability in the width of the Z-line is known to be caused by mutations in several of the constituent proteins. Visual inspection of the electron micrographs clearly indicates that the Z-disks from the MYBPC3 E542Q sample are wider compared to the myectomy control, and also considerable heterogeneity in the width is observable. With the MYBPC3 HCM sample, again a regional variation in Z-band area per unit Z-disk length was measured; 78 µm in area 2 and 107 µm in area 4, whilst the Z-band area per unit Z-disk length for the myectomy control (no identified mutation) was much smaller (50 µm).

Methodology developed for skeletal muscle analysis has enabled study of normal human heart muscle samples. We have calculated an electron tomogram of rat cardiac muscle Z-band. It shows clear links due to alpha-actinin between anti-parallel actin filaments that overlap in the Z-band. It also shows a putative structure assigned to Cap-Z.

Deliverables

D5.1 Report on the model of the arrangement of cMyBP-C on cardiac thick filaments (month 30).

WP6 Interventional approaches in models of HCM

Start month: 1

End Month: 42

WP Leader: P4 UKE

Task 6.1 The role of metabolic modulation in ameliorating energetic deficiency in HCM

This task aimed to test whether manipulation of cardiac metabolism by perhexiline (a compound that increases cardiac energetics by causing a switch from fatty acid to glucose metabolism) in a mouse model of HCM is able to ameliorate energetic deficiency and progression of thesease. The initial work set out to identify a suitable mouse model in which to carry out the investigation, and it was first proposed to use an inducible TNNT2 transgenic mouse model developed by CO1 UOXF.HS. The expression of the transgene was confirmed at the RNA level with the FLAG-tagged human cTnT protein also being detectable by Western blot. However, the level of protein was unexpectedly low and therefore not sufficient to cause cardiomyopathic changes in the heart. Four further mouse models were examined for their suitability in pharmacological intervention studies of which two were identified for further work: a Mybpc3-targeted knock-in (KI) model mimicking a human MYBPC3 mutation (from P4 UKE), and an inducible ACTC1 E99K transgenic mouse model.

Mybpc3-targeted KI mice were treated with perhexiline or placebo and underwent intensive in vivo and ex vivo cardiac phenotyping (echocardiography, invasive hemodynamic measurements, morphometric measurements, RT-qPCR). Perhexiline levels equivalent to human therapeutic dose (&gt; 0.15 mg/L) were achieved in 5 out of 14 treated animals. Cardiac performance did not improve in treated animals; however animals with therapeutic perhexiline levels showed a reduced calculated LV mass. RT-qPCR revealed no significant difference in the mRNA levels of parameters of hypertrophy (Acta1,
Myh7, Nppa, Nppb), however trends towards reversal of the HCM phenotype were evident for collagen expression (marker of fibrosis) and Myh7 to Myh6 ratio. Thus perhexiline treatment did not reverse the severe HCM phenotype, although there was evidence of partial reversal of individual parameters (e.g. LV mass and fibrosis). Longer treatment, higher doses, alternative drugs or an earlier start of the treatment may be required.

Additional insight may be obtained in an inducible model in which the onset of disease (and the effect of pharmacological intervention on it) may be strictly controlled. Thus inducible ACTC1 E99K transgenic mouse were generated by crossing responder and inducer lines. Double-transgenic female animals (DBT) were fed with inducing agent erythromycin for 3 weeks and transgenic protein detected with a specific ACTC1-E99K antibody. Transgenic expression was detectable in both treated DBT, however there is also evidence of leaky expression in one of the non-treated DBT animals. No SCD events were observed in animals expressing the mutant protein. The novel inducible mouse model of HCM shows expression of mutant sarcomeric protein in response to erythromycin. After optimisation to overcome leaky expression and to increase expression levels, this model will be a valuable tool to study acute mechanisms of HCM and to test therapeutic interventions such as perhexiline. However, the model is unlikely to gain insights in SCD events.

Task 6.2 Role of serotonin and reactive oxygen in modulating human fibroblast growth

The first step of Task 6.2 was to set-up cultures of human ventricular fibroblasts from HCM and control samples. HCM fibroblasts showed diverse morphology and a very slow growth (confluence was reached approximately after 2 weeks in culture). This was somehow unexpected, due to the lack of information in the literature. This delayed the next step. In addition, evaluation of the involvement of reactive oxygen species (ROS) was discouraged by the paucity of available cells.

In a second step, we investigated the molecular and functional properties of HCM fibroblasts. The effects of a number of factors on the modulation of cell growth were tested, using fibroblast growth factor as a positive control. We found that angiotensin II and α-methyl-serotonin exerted a clear stimulating effect on fibroblast growth, suggesting a role of the pathway downstream the AT1 and 5-HT2 receptors in the development of myocardial fibrosis in HCM. Interestingly, whereas mRNA level of 5HT2B receptors was higher in ventricular and septal tissues from HCM than from control individuals, no difference was detected in isolated fibroblasts form both groups. Stimulation of fibroblasts with α-methyl-5HT, a selective 5-HT2 agonist, elicited a marked response as documented by appearance of Ca2+ transients due to release from intracellular stores. 5HT2 receptor stimulation did not alter HCM fibroblast proliferation. In addition, collagen 1A1 mRNA level was higher in HCM than in control fibroblasts, suggesting fibroblast activation. Interestingly, while stimulation with angiotensin II caused a significant increase in collagen 1A1 in control fibroblasts, no further increase in mRNA level was observed in HCM fibroblasts, suggesting dysregulation of signaling pathways controlling collagen synthesis. Thus, HCM fibroblast in culture may represent a suitable model to investigate the molecular mechanisms underlying the development of myocardial fibrosis and their pharmacological control.

Task 6.3 Evaluation of the relative contribution of UPS and NMD in human HCM

Mutant proteins resulting from MYBPC3 frameshift mutations were never detected in human HCM. Previous findings of P4 UKE suggest that nonsense-mediated mRNA decay (NMD) and ubiquitin-proteasome system (UPS) regulate the expression of a point mutation in Mybpc3-targeted KI mice with HCM [28]. The involvement of these systems in human HCM was not known.

Task 6.3 aimed at establishing an in vitro screening system in HEK293 cells to evaluate the relative contribution of UPS and NMD in the regulation of expression of human mutant MYBPC3 minigenes. We focused on two mutations that should result in C-terminal truncated proteins. The major findings are that HEK293 cells stably expressing human MYBPC3 minigenes are suitable to evaluate the expression of mutations at the level of mRNA and reproduce data obtained in humans. This study provides the first evidence that stable HEK293 cells can be used for screening the expression of human MYBPC3 mutations.
We also evaluated more deeply the contribution of the UPS and of the second major proteolytic pathway, which is the autophagy-lysosomal pathway (ALP) in HCM. The major findings are as follow: i) adrenergic stress induced UPS impairment (reduced chymotrypsin-like activity of the proteasome) only in heterozygous Mybpc3-targeted KI mice that carry a human HCM mutation, but not in heterozygous Mybpc3-targeted knock-out; ii) the global activity of the proteasome was impaired (accumulation of the UPS substrate UbG76V-GFP) with aging in homozygous Mybpc3-targeted KI mice. Taken together, these data suggest that combination of stress (adrenergic or aging) and mutation is sufficient to impair the UPS [29, 30].

More recently, we also evaluated UPS and ALP in ventricular samples of patients with HCM (collaboration P4 UKE - P2 VUMC). We included myocardial samples from 8 patients with MYBPC3 mutations (M3-positive) and 10 patients without identified mutations (sarcomere-negative). The major finding is a 70% lower level of poly-ubiquitinated proteins in M3-positive than in sarcomere-negative patients, whereas the chymotrypsin-like activity of the proteasome did not differ between groups. These data did not allow drawing a clear conclusion of whether the UPS is impaired or not in M3-positive patients. Further analysis with controls (non-failing samples) is needed.

Task 6.4 Evaluation of spliceosome-mediated RNA trans-splicing as a tool for gene therapy of HCM

Current pharmacological treatment of HCM such as beta-blockers can help to reduce the stiffness in the thickened heart but are not effective in reversing cardiac hypertrophy. Therefore, the main goal of Task 6.4 was to target the underlying mechanisms of HCM by using RNA-based therapy.

We evaluated whether spliceosome-mediated RNA trans-splicing can be used as a novel therapeutic strategy for HCM in the Mybpc3-targeted KI mice [28]. This mouse carries a point mutation (G&gt;A transition) on the last nucleotide of exon 6, which results in low levels of mutant Mybpc3-mRNAs and cMyBP-C-proteins. 5'-trans-splicing was induced between mutant endogenous Mybpc3 pre-mRNA and the pre-trans-splicing molecule (PTM) carrying a FLAG-tagged wild-type Mybpc3 cDNA sequence. PTMs (with or without polyA signal) were packaged into adeno-associated virus for transduction of cultured cardiac myocytes (serotype 6) and the heart in vivo (serotype 9). Full-length repaired Mybpc3 mRNA represented up to 66% of total Mybpc3 transcripts in cardiac myocytes and 0.14% in the heart. Repaired cMyBP-C protein was detected by immunoprecipitation in cells and in vivo and exhibited correct incorporation into the sarcomere in cardiac myocytes. This study provides i) the first evidence of successful 5'-trans-splicing in vivo and ii) proof-of-concept of mRNA repair in the most prevalent cardiac genetic disease [31].

We also evaluated the exon skipping strategy. Exon skipping mediated by antisense oligoribonucleotides (AON) is a promising therapeutic approach for genetic disorders, but has not yet been evaluated for cardiac diseases. We investigated the feasibility and efficacy of viral-mediated AON transfer in the Mybpc3-targeted KI mice. We identified an alternative variant (Var-4) deleted of exons 5-6 in wild-type and KI mice. To enhance its expression and suppress aberrant mRNAs we designed AON-5 and AON-6 that mask splicing enhancer motifs in exons 5 and 6. AONs were inserted into modified U7 small nuclear RNA and packaged in adeno-associated virus (AAV-U7-AON-5+6). Transduction of cardiac myocytes or systemic administration of AAV-U7-AON-5+6 increased Var-4 mRNA/protein levels and reduced aberrant mRNAs. Injection of newborn KI mice abolished cardiac dysfunction and prevented left ventricular hypertrophy [32].

Since current therapeutic options for HCM only alleviate symptoms, these two different proof-of-concept studies open new horizons for causal therapy of the severe forms of the disease.

Deliverables

D6.1 Report on the efficacy of spliceosome mediated RNA transsplicing (SmaRT) strategy (Task 6.2) for mediating changes to MYBPC3 mRNA in vitro (month 18).
Task 7.1 Characterization of the pre-hypertrophic phenotype of HCM

The concerted efforts of BIG-Heart and other researchers have described the predictors of HCM disease progression from the classic phenotype to the end-stage phase. These data have led to the proposal that disease progression and development of LV dysfunction can be accurately predicted in HCM patients, years before the actual clinical demise, allowing time for preventive measures [33]. In the light of this, we have constructed a clinical score ('BIG-Heart HCM Score') for prediction of disease progression, upon which clinicians can tailor follow-up strategies in HCM patients following diagnosis. The score, ranging from 0 to 22, was constructed by attributing an arbitrary score to 12 clinical or instrumental items identified in the literature, and validated on a large HCM patient cohort at P3 UniFl. A score 0-4, judged to reflect low risk, was present in 112 patients (61%), a score 5-8, judged to reflect medium risk, in 58 (32%), and a score 9-22, consistent with high risk, in 13 (7%). Prevalence of the study endpoints (SE) was 1%/year in patients with scores 0-4, 6%/year in patients with scores 5-8 and 22%/year in patients with scores 9-20. Multivariable logistic regression survival analysis showed that each unit increase in the score was associated with a 5% increase in likelihood of heart failure (HF)-related events (hazard ratio 1.07 p=0.01). Negative predictive accuracy of a score <4 was 92%; positive predictive accuracy of a score >8 was 68%.

Based on these data, it is plausible to provide tailored indications regarding follow-up in patients based on their baseline 'BIG-Heart HCM Score' (to be reassessed serially at each visit):

• Score 0-4: re-evaluate at 1-2 years (except when considering myectomy or primary anti-arrhythmic prophylaxis)
• Score 5-8: re-evaluate at 1 year, maintain close surveillance by imaging, control of risk factors for progression (e.g. atrial fibrillation).
• Score 9-22: assess every 6 months, aggressive HF treatment and prevention of sudden cardiac death.

The 'BIG-Heart HCM Score' provides clinicians with a score capable of tailoring such evidence to the individual patient and identify subgroups at different risk of disease progression and HF-related complications. It is hoped that the results of the prospective applications of the score, which go beyond the span of the present project, will confirm its accuracy and clinical utility in HCM cohorts. Potential spin-offs include the possibility of creating comparable patient categories for clinical trials, implementation of timely prevention with novel pharmacological approaches or devices.

Task 7.2 Assessment of microvascular function in patients with sarcomere gene positive and negative in HCM

Comparison of coronary flow response to dipyridamole by positron emission tomography (PET)-ammonia revealed that HCM patients with myofilament mutations are characterized by more severe impairment of micro-vascular function and increased prevalence of myocardial fibrosis, compared to genotype-negative individuals. These findings suggest a direct link between sarcomere gene mutations and adverse remodelling of the microcirculation in HCM, accounting for the increased long-term prevalence of ventricular dysfunction and heart failure in genotype-positive patients. Further work has been directed at assessing whether, and to what extent, the type of sarcomere filament involved might account for the clinical heterogeneity observed within the large genotype-positive subset of HCM patients, possibly reflecting different magnitude of energetic and micro-vascular abnormalities. Specifically, we directed our interest to the subgroup of patients harbouring mutations of sarcomere thin filament protein genes (cardiac troponin T and I, tropomyosin and cardiac actin), whose phenotype and clinical
course, compared to the more prevalent thick filament-related form, is unresolved. We therefore characterized a multi-centre cohort of 84 patients with thin filament-related HCM, followed for an average of 5 years, compared to 157 HCM patients with thick filament-related disease. We found that patients with thin filament HCM showed: (i) lesser maximal LV wall thickness values (18±5 mm vs. 24±6 mm, p<0.001) with larger prevalence of atypical distribution of hypertrophy; (ii) higher rate of adverse events during follow-up, including cardiovascular death, resuscitated cardiac arrest, appropriate ICD shock, nonfatal stroke or progression to NYHA class III or IV (annual event rate 4.6% vs. 2.7%, p=0.032); (iii) higher likelihood of progression towards LV systolic dysfunction (left ventricular ejection fraction <50%) and/or restrictive diastole, the so-called end-stage of HCM (30% vs. 18%, p=0.02). Furthermore, patients with thin filament HCM had 2.2 fold higher prevalence of triphasic left-ventricular filling pattern (26% vs. 12% in thick filament HCM, p<0.001). Thus, HCM related to thin filament mutations is characterized by significant differences in phenotype and clinical course compared to the more prevalent thick-filament HCM, including higher risk of adverse events related to ventricular arrhythmias as well as progressive LV dysfunction. Triphasic LV filling is distinctively common in thin filament HCM, pointing to peculiar molecular determinants of diastolic dysfunction in this genetic subset. Further studies are required to understand the molecular, energetic and micro-vascular correlates of these findings, in hopes of identifying gene-specific targets for pharmacological treatment of HCM.

Task 7.3 The role of metabolic modulation in preventing exercise-induced stunning in HCM

HCM patients have impaired myocardial energetic status that correlates significantly with exercise capacity. Although we have now shown that energy deficiency in HCM does not frequently manifest as exercise-related myocardial stunning, we have confirmed a robust relationship between energy deficiency and exercise in HCM. Furthermore, metabolic modulation with perhexiline can improve exercise capacity in HCM [34]. In order to delineate the metabolic determinants for exercise incapacity in HCM and the influence of perhexiline on them, we collected blood samples from coronary sinus, femoral vein and aortic root, during different physiological states (rest, after exercise and after pacing the heart). Metabolomic analysis is being performed on these samples using mass spectrometry and NMR spectroscopy. The control group has been recruited and a detailed assessment of their cardiovascular physiology carried out using ECG, ECHO, CPEX (Cardiopulmonary exercise test) and blood tests. Recruitment and analysis of the HCM cohort are nearing completion and the data will reported shortly after the end of BIG-Heart.

Deliverables

D7.1 Report on the suitability of the described integrated diagnostic approach (task 7.1) in the characterization of the early HCM phenotype (month 30).


WP8 Dissemination

Start month: 1

End Month: 42

WP Leader: CO1 UOXF.HS

Since the beginning of the project, the dissemination of results has been considered a key factor for the success of the project. This activity has been based on a strategy and articulated in a plan that has allowed the partners to achieve a dissemination of results and outcomes on a continuous basis. Internal dissemination has been ensured by emails and regular newsletters. The external dissemination activity has been oriented both at the national level of the partners and at the European level to
inform other stakeholders and participants within the cardiovascular sector.

The objectives of the dissemination were:

Promoting the visibility of interim and final results through scientific dissemination by means of web-site, leaflet, publications and participation to events; identifying the most suitable target groups.

Key achievements of this WP were:

• Logo;
• The design and implementation of the web-site (www. http://www.big-heart.eu/ );
• Leaflet;
• Papers and publications with a range of impact factor from 1.358 to 51.658. In details, 43 oral presentations, 23 invited lectures, 57 posters, 9 oral communications and 4 published abstract have been made. 57 publications, 1 of which in press, published in 29 scientific journals have been produced during the period.
• 42 conferences;
• 3 symposia;
• 10 seminars;
• 3 international workshops.
• one TV interview and open days to engage with the general public

Contributing partners: CO1 UOXF.HS P6 CFc and all partners

WP9 Management

Start month: 1

End Month: 42

WP Leader: CO1 UOXF.HS

This WP has carried out the overall project management, ensuring that a proper co-ordination across tasks and across partners was maintained, to achieve the project goals within the time and budget constraints.

According to Art. II.6.5 project management activities included: the overall legal, ethical, financial and administrative management, the maintenance of the Consortium Agreement as well as the other management activities, foreseen by Annex I (such as communication with the European Commission, administration of the financial contribution, reporting and organisation of project meetings).

Objectives of this WP were: to co-ordinate the various project components (research activity, legal, administrative and logistic issues); to assure a timely reporting of the project to the EC; to establish and adopt common operational procedures; to monitor, track and control the progress of the project, the costs, financial and scheduling changes; to ensure respect of ethical issues and attention paid to gender issues; to ensure high quality of scientific and technical results, verifying that each report covers all aspects required to meet the deliverables; maintenance of the Grant Agreement and of the Consortium agreement; overall legal, ethical, financial and administrative management; any other management activities as below described.

Key achievement of this WP were:
- Distribution of pre-financing and periodical payment in line with Grant Agreement;
- Periodical collection of financial and scientific data in order to monitor project status and to pave the way toward official reports;
- Preparation of the periodical report at month 18 and 42;
- Preparation of 3 amendment requests including one for project extension;
- Monitoring of achievement of milestones and deliverables;
- Assessment of deliverable contents.
- Organisation of annual meetings and/or teleconferences;
- Ensuring smooth communication among partners.

Contributing partners: CO1 UOXF.HS and P6 CFc

Potential Impact:

Through our described hypothesis-driven multidisciplinary approach, BIG-Heart aimed to obtain a deeper understanding of HCM and to pave the way for novel therapeutic avenues. Both may be of future benefit for a variety of stakeholders, e.g. for the scientific community, for health authorities, for practising clinicians, for patients suffering from HCM and other cardiac diseases and for SMEs as well as for pharmaceutical industries.

In the field of science and technology, BIG-Heart targeted the following aspects: the project aimed (i) to gain deeper mechanistic insights into the disease pathology of HCM, (ii) to develop novel methodologies in experimental and clinical sciences, (iii) to explore novel therapeutic avenues in laboratory test settings and for patient treatment and (iv) to improve tools to clinically characterise and classify patients, in order to customise their treatment.

(i) Deeper insight mechanistic insight into the disease pathology of HCM

The multi-disciplinary approach of all 5 BIG-Heart centres has not only expanded existing theories of how HCM is caused by genetic mutations, but more importantly has additionally provided novel concepts: BIG-Heart activities have confirmed and expanded investigations using a large number of patient samples, and revealed alterations in cardiac structure, calcium handling, cellular energetics, excitation-contraction coupling, protein expression and phosphorylation. Of note, novel findings were e.g. the uncoupling of calcium sensitivity from troponin phosphorylation, the detailed analysis of electric abnormalities in HCM patient material and mouse models explaining the risk of cardiac arrhythmias, and the detection of inefficient energy usage in HCM. Furthermore, the comparison of HCM with other forms of cardiac disease (e.g. heart failure, dilated cardiomyopathy or left ventricular hypertrophy caused by aortic stenosis) has allowed a dissection of primary "HCM-specific" effects from secondary changes in the heart of patients.

(ii) Development of novel methodologies in experimental and clinical sciences

A unique resource of BIG-Heart is a tissue collection of more than 300 cardiac samples. The majority of these samples are from HCM patients, but also samples from patients with other cardiac abnormalities and reference samples (controls) were obtained. The consortium has created a database to manage the samples and relevant information, and to communicate associated research findings. Extensive sample acquisition has taken place at four centres and all centres have exchanged samples to enhance the potential for all research activities involving patient material. Moreover, the large number of samples allows correlation of experimental or clinical HCM parameters with the underlying gene mutation.

Animal models, incorporating the genetic mutations found in HCM patients, have been generated and used as part of the BIG-Heart activities. Mouse models allow the invasive interrogation of cardiac HCM phenotypes in vivo. Furthermore, treatment experiments can generate preliminary data justifying the preparation of clinical trials for established or novel drugs.
particular, the ACTC1 E99K mouse model reflects arrhythmic aspects of HCM and has extensively been studied in the project. Treatment of established HCM mouse models with drugs (e.g. perhexelinel and ranolazine) has gained insights into their mode of action and has allowed us to evaluate their potential for clinical trials. In addition we are generating and testing animal models where the HCM mutation can be switched on at later stages in life. Such mouse models will help to get further insight into HCM disease mechanisms and assist us in dissecting primary from secondary changes in disease.

Cellular test systems have been established to complement the above research activities. On the one hand, methods have been developed to isolate cells form HCM patients, using the unique BIG-Heart tissue collection. Such cells provide valuable insights into the pathological changes observed in HCM, and both cardiac cells and non-cardiac cells (fibroblasts) have been studied extensively. However, the project has also developed strategies to model the disease situation in the absence of available patient material: cellular test systems have been set up to understand the consequences of MYBPC3 mutations. In addition we have designed methods to exchange mutant proteins, which have been generated in the laboratory, into a test cellular or in vitro setup (e.g. in vitro motility assays), thereby mimicking the protein setup in the HCM patients.

(iii) Exploration of novel therapeutic avenues in laboratory test settings and for patient treatment

Conventionally, the clinical management and pharmacological therapies in HCM focus on the alleviation of symptoms (e.g. removal of outflow tract obstruction by myectomy). A major component of the BIG-Heart activities has been the development of novel therapeutic approaches beyond alleviating symptoms, in order to provide specific therapies for HCM, which are based on our novel insights in disease mechanisms. Spliceosome-mediated RNA trans-splicing (SmaRT), a new tool for RNA-based therapy of genetic diseases, has successfully been employed to “repair” a HCM-causing MYBPC3 mutation in cells and a mouse model. Importantly, this approach has improved cardiac function in the animal model, hence being a promising concept which could be explored for application in humans. For a second RNA-based approach, the exon skipping strategy, the proof-of-concept was provided in cellular test setups and future research will focus on its further optimisation and application.

BIG-Heart has provided convincing experimental evidence for beneficial effects of the drug ranolazine in HCM. In cardiomyocytes from HCM patients, it could be shown that the drug is able to reverse pathological arrhythmic changes and animal work is currently carried out to test the drug’s efficiency in ameliorating the HCM phenotype in mice. Once sufficient experimental evidence is accumulated, the application of the drug in the therapy of human HCM will be explored.

Moreover, energetic modulation of the HCM phenotype has been tested in animals and patients using the drug perhexilinel. Again, the work in mouse models has allowed to gain detailed into the mode of action of the drug and the clinical work in HCM patients has shown symptomatic improvement. A wider application of the drug in the clinical management of HCM will be tested in future clinical trials.

(iv) Improved tools to clinically characterise and classify patients

The wider application of high-throughput sequencing (“next generation sequencing”) in clinical practice coincided with the start of the BIG-Heart project. This made genetic information for the majority of HCM patients more readily available and has been a major step forward in the clinical characterisation of patients. Now correlations of genotypes with phenotypes are feasible on a wide scale, and BIG-Heart has contributed to the understanding of the different clinical entity of HCM patients with thin filament mutations. These patients are at higher risk of developing heart failure and have a higher occurrence of arrhythmic events, hence requiring closer clinical follow up than patients with thick filament mutations.

In addition, genetic information is now often available for young individuals before the development of a HCM phenotype. This allows continual follow up and early intervention at the onset of disease. In this context, the BIG-Heart HCM score has been developed, providing a tool for the risk stratification of HCM patients and tailoring their clinical follow up appropriately. This score may not only provide better disease classification, it may also reduce costs in health care services, as low risk HCM
patients will have less frequent follow up, while high risk patients will be treated efficiently to prevent heart failure related emergency interventions.

Other activities of BIG-Heart have focused on the characterisation of microvascular and valve dysfunction in HCM patients, demonstrating that not only the cardiac muscle, but also other structural components of the heart are affected by the disease. Future research may provide future insights into these abnormalities, which are currently poorly understood and hence can only be treated symptomatically.

Impacts on the scientific community:

During its entire course, BIG-Heart has contributed to the accumulation of knowledge in the scientific community, and several key points of this advancement of knowledge have been highlighted in the above section.

Dissemination of the advancement of knowledge has been an important activity of the consortium: Within the consortium, regular meetings, newsletters/emails and conference calls have been used to inform partners of the progress of the Science and Technology activities.

Scientific seminars and symposia have been organised by BIG-Heart partners to coincide with the BIG-Heart meetings open to young researchers of all partner organisation, in addition to external researchers.

Newsletter/emails have been regularly distributed (approximately one a month) to all partners (sent to the Principal Investigators to cascade in their groups) in order to notify participants about the project’s progress and to inform them of forthcoming deadlines (e.g. deliverables) and meetings. BIG-Heart newsletters/emails were prepared in English and were edited by the project manager.

The private section of website has been used as a tool to communicate and disseminate meeting presentations, methods, protocols and deliverables internally and the BIG-Heart database, which was available online to all partners, has proved to be an efficient tool to coordinate and disseminate activities related to the tissue collection.

Engagement with the scientific community has occurred at local, national, European and international levels.

The publication of results in scientific journals and the participation in conferences and symposia have both been key components of the dissemination strategy.

BIG-Heart has been widely presented in international conferences (42), workshops (3), and also in 10 seminars (out of which 1 invited seminar), 3 Symposia. 43 oral presentations, 23 invited lectures, 57 posters, 9 oral communications and 4 published abstracts have been made. 57 publications, published in 29 scientific journals have been produced. The publications report the following range of impact factors: from 1.358 to 51.658

The project website has been periodically updated, to present and disseminate information about BIG-Heart to the scientific and clinical community and the general public and as an instrument for information exchange and sharing among the partners.

A leaflet, devoted to support the presentations at events and the individual meetings, has been distributed. It has also served as a communication tool for potential mailings to target audiences.

An important impact of the BIG-Heart project has been the enrichment within the scientific community by promoting the careers of young scientists. Many members of the BIG-Heart consortium are at the beginning of their scientific career
(graduate students, PhD students, postdoctoral researchers; referred to as “early career researchers”) and have benefited from the world-leading research environment, state-of-the art equipment in the facilities, the expertise of experienced researchers and the exchange of knowledge, expertise and experience within the consortium. Emphasis has been put on actively involving the early career researchers in internal and external dissemination activities. E.g. they were encouraged to attend BIG-Heart meetings and at least two of each centre were given the opportunity to present research findings at each BIG-Heart meeting. Moreover, they participated actively in external dissemination activities such as oral and poster presentations at scientific conferences. Importantly, they are often first authors on numerous publications in scientific journals, stemming from BIG-Heart activities. Such publications enhance their visibility and employability in the scientific community and the success of the strategies to promote early career researchers is measurable: Some of the early career researchers have secured attractive research positions in research groups of international reputation following their employment on BIG-Heart or have obtained competitive fellowships of national and international relevance.

Women are still under-represented in senior research positions (e.g. Principal Investigators) within the European research community. BIG-Heart has contributed to the promotion of female scientists, mainly by providing role models. Two of the Principal Investigators (Prof. Jolanda van der Velden, P2 VUMC/P7VU/Umc and Prof. Lucie Carrier, P4 UKE) are female professors with internationally renowned research reputation, both securing their full professorships during the period of BIG-Heart. Their presence in the consortium has encouraged female scientists to apply for positions within BIG-Heart. Similar to early career researchers, female scientists were actively encouraged to participate in internal and external dissemination activities and have benefited from the research outputs of BIG-Heart in terms of their visibility within the scientific community.

The competiveness and high-quality research output of the BIG-Heart laboratories and their visibility within the scientific community makes the research groups attractive partners for future grant applications. All centres are involved in new grant applications at local, national, European and international level. The BIG-Heart consortium liaised with additional scientific groups and SMEs to apply for EU grants and will continue with such activities in the future.

Impacts on the general public and on HCM patients:

The efficient dissemination strategy has intended to raise awareness of the general public for BIG-Heart’s activities and to communicate the progress in the advancement of knowledge. The website has been used to relay information about HCM and the BIG-Heart activities in lay terms in 14 European languages, and the leaflet has been distributed at meetings and conferences specifically in areas where patient organisations exhibit their activities with the intention that lay public and HCM patients will have access to the information.

TV interviews and open days at partners’ clinical facilities were specifically aimed at engaging with the public and to explain research activities and outcomes in lay terms.

BIG-Heart S&T activities might significantly impact on the clinical care and treatment of HCM patients in the future: Many findings of the project improve the understanding of the mechanisms underlying the disease and hence are the prerequisite for more specific therapeutic approaches. E.g. integrating genetic information into the research activities may lead to genotype-specific therapies for individuals with HCM. There is substantial progress in the understanding and treatment of life-threatening arrhythmias. This may prevent sudden cardiac death events associated with HCM in future and reduce the costs in health care systems, e.g. by replacing costly implantation of Implantable Cardioverter-Defibrillators (ICDs) with cost-efficient pharmacological therapies.

In addition, patients will benefit from a better clinical characterisation, using the “BIG-Heart HCM Score” to risk-stratify HCM patients. For individuals with low risk, the benefit may arise from less frequent and less invasive follow ups, while high risk patients may benefit from closer follow up and tailored therapies to prevent development of heart failure.
Of note, there are several promising therapeutic approaches, which were developed and enhanced by BIG-Heart S&T activities. Spliceosome-mediated RNA trans-splicing (SmaRT) has been shown to be a novel tool to “repair” HCM mutations in cellular test setups and animal models. If it can be applied to humans, HCM patients may benefit from this new therapeutic approach in the long run. Moreover, the demonstration that ranolazine can reverse arrhythmic aspects of HCM will justify clinical trials and hence might lead to the application of the drug for the treatment of HCM. Likewise, BIG-Heart’s work on the benefits of perhexiline in alleviating energy deficiency in HCM may support further testing of the drug’s potential in the treatment of HCM patients.

In summary, the project has highlighted the potential of two established drugs, where the safety has already been documented, plus a completely novel approach (SmaRT) as potential novel therapeutic option for HCM patients. This would be the first treatment options based on insights into disease mechanisms of HCM.

Potential impact on private sector industries and SMEs:

The pharmaceutical industry may benefit from the commercial exploitation of the two drugs evaluated in the project, as well as from the exploitation of novel methodology and therapeutic approaches. So far, only informal discussions have taken place between project partners and pharmaceutical industry; the BIG-Heart consortium has decided to consolidate research findings first before aiming to commercialise them.

The competitiveness and visibility of BIG-Heart within the scientific community has made the research groups attractive partners for European private sector companies and SMEs. E.g. the SME IonOptixs benefits from the expertise of P2 VUMC/P7 VU/VUmc in bilateral collaborations, while the SME PolyGene has established links with CO1 UOXF.HS and P5 Imperial.

The productivity of these interactions was reflected in the active participation of three private sector companies and SMEs in joint grant applications of the BIG-Heart consortium.

In summary, the BIG-Heart project has immensely contributed to the advancement of knowledge in the understanding and treatment of HCM, it has had substantial positive impact on the scientific community and there is potential benefit also for the general public, HCM patient and private sector industries as well as SMEs.

List of Websites:

Project website: http://www.big-heart.eu/

Relevant contact details:
Dr. Charles Redwood - The Chancellor, Masters and Scholars of the University of Oxford
Tel: +44 1865 234661
Fax: +44 1865 234667
E-mail: credwood@well.ox.ac.uk

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