Chapter 1

General introduction and thesis outline

List of abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
Ca^{2+}	calcium
CK	creatine kinase
cMyHC	cardiac myosin heavy chain
cTm	cardiac tropomyosin
cTn	cardiac troponin
cTnC	cardiac troponin C
cTnI	cardiac troponin I
cTnT	cardiac troponin T
HCM	hypertrophic cardiomyopathy
IVS	interventricular septum
MLC-1/2	myosin light chain 1 / 2
MYH7	gene encoding cardiac myosin heavy chain
MYBPC3	gene encoding cardiac myosin binding protein C
Na^{+}	sodium
P_i	inorganic phosphate
PKA	protein kinase A
SR	sarcoplasmic reticulum
SERCA	sarcoplasmic reticulum calcium ATPase
TPM1	gene encoding cardiac tropomyosin
TNNC1	gene encoding cardiac troponin C
TNNI3	gene encoding cardiac troponin I
TNNT2	gene encoding cardiac troponin T

Familial hypertrophic cardiomyopathy

It was back in 1951 when a young boy of 14 years old suddenly collapsed on the playground of his school after being chased. When he arrived in the hospital he already passed away. During his life he had a history of dizziness, a third heart sound and a soft systolic murmur. The advice he received from the doctor was to live a normal life, however without excessive sports. Interestingly, when his heart was studied after his death the interventricular septum (IVS) was hypertrophied and the muscle fibers were not arranged in parallel as observed in healthy hearts, but merely ran in diverse directions.[1]

This patient was described in an article by doctor Donald Teare in 1957, one of first thorough descriptions of a patient who most likely suffered from hypertrophic cardiomyopathy (HCM). Since 1979 the terminology HCM has been widely accepted for the disease.[2] The most recent definition of HCM positioned by the European Society of Cardiology is:

A myocardial disorder in which the heart muscle is structurally and functionally abnormal, in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease sufficient to cause the observed myocardial abnormality.[3]

The incidence of HCM is 0.2% of the general population and the clinical phenotype, indeed, is characterized by asymmetrical left ventricular (LV) thickening of the IVS and cardiomyocyte disarray.[4] In addition to the large incidence of the disease, HCM is the most common cause of sudden cardiac death in competitive athletes, at least in the United States.[5] An important physiological characteristic of an athlete is an enlarged heart to meet the haemodynamic challenges due to excessive sport activities. However, this physiological LV wall thickening is in some cases hard to distinguish from pathological hypertrophy.[6] The main cause of HCM is thought to be a genetic (DNA) defect inherited via an autosomal dominant pattern. Currently, 13 different genes are associated with familial HCM, which most frequently encode for sarcomeric proteins. To understand how a genetic defect may lead to cardiac disease, it is important to know some basic principles regarding cardiac and sarcomere contraction.

The heart and circulation

The heart consists of a right and left part, which connect the small (pulmonary) and large (systemic) circulation. From the right atrium blood flows to the right ventricle from where it is pumped to the lungs and oxygenated. Subsequently the oxygen-rich blood flows to the left side of the heart, into the systemic (body) circulation, via the left atrium and ventricle. Atrial and ventricular contraction, which contribute to blood flow through both circulations, exist of two phases: diastole and systole. During diastole, the relaxation phase, the cavities are filled with blood from the veins. Subsequently, during systole, atrial contraction contributes to the filling of the ventricles followed by ventricular contraction and ejection of the blood into both circulations.

Cardiac muscle structure

The heart is composed of cells which take care of heart rhythm (pacemaker and conduction system), and cells which are built to contract, the cardiac muscle cells; also called cardiomyocytes. The smallest functional contractile unit of the cardiomyocyte is the sarcomere consisting of thick and thin filament proteins. The organization of these thick and thin filament proteins results in the typical striated pattern (Figure 1).

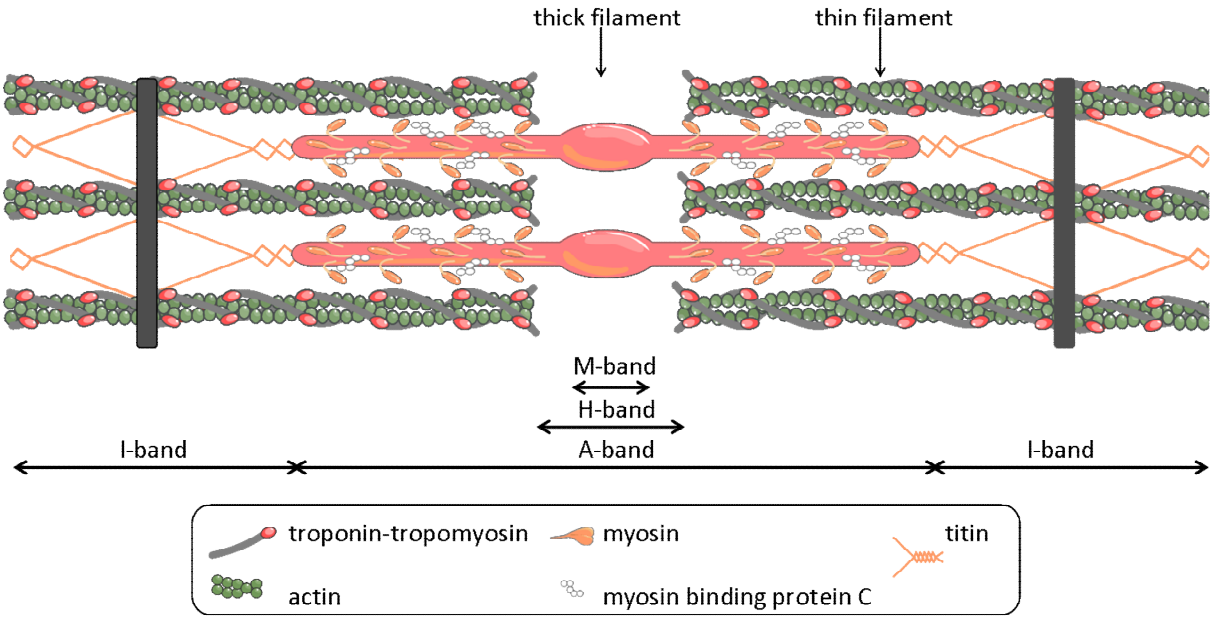

Figure 1. Schematic representation of striated muscle components. The organization of sarcomeres with the typical striated pattern of thick and thin filament proteins.

The main thick filament protein is myosin II, a motor protein. This is a dimer protein, consisting of two myosin heavy chains (cMyHC) and two pairs of myosin light chains (MLC-1 and MLC-2). Each cMyHC contains a globular head (S1 domain), a hinged S2 domain and a rod domain (Figure 2A). The S1 domain holds the adenosine triphosphate (ATP) binding site and the hinged S2 domain the binding sites for MLC-1 and MLC-2. The cMyHC rod forms the core of the thick filament.[7,8] There are two different cMyHC isoforms. In the human ventricle approximately 95% is the slow (more efficient) β-MyHC and the remainder is the fast α-MyHC.[9]

Another thick filament protein is cardiac myosin binding protein C (cMyBP-C, Figure 2B) which consists of eight immunoglobulin domains, a proline/alanine rich domain, three fibronectin type III domains and a unique regulatory sequence: the M-domain. The N-terminal domains C0-C2 are thought to interact with actin[10] and the C1-C2 domains with the S2 domain of cMyHC, both in a phosphorylation-dependent manner.[11] The C-terminal domains C7-C9 of cMyBP-C are important for the correct insertion of the protein into the entire thick filament,[12] which might be controlled by the interaction of the C8-C10 domains with titin.[13] cMyBP-C is arranged partially perpendicular to the thick filament surface and reaches neighboring thin filaments.[14] The thick filament proteins are located in the middle of

the sarcomere, collectively called the A-band. The M-band exists of proteins connecting the thick filaments.

The thin filament is mainly composed of actin, the cardiac troponin (cTn) complex and cardiac tropomyosin (cTm) (Figure 1). cTn consists of three subunits: cardiac troponin C (cTnC), troponin I (cTnI) and troponin T (cTnT). The entire troponin complex is linked to cTm via cTnT and Tm is connected to actin. Each sarcomere contains two sets of thin filaments. The thin filaments are connected to intermediate discs proteins, collectively called the Z-disc. The giant spring-like protein titin links the Z-disc to the M-band.[7,8]

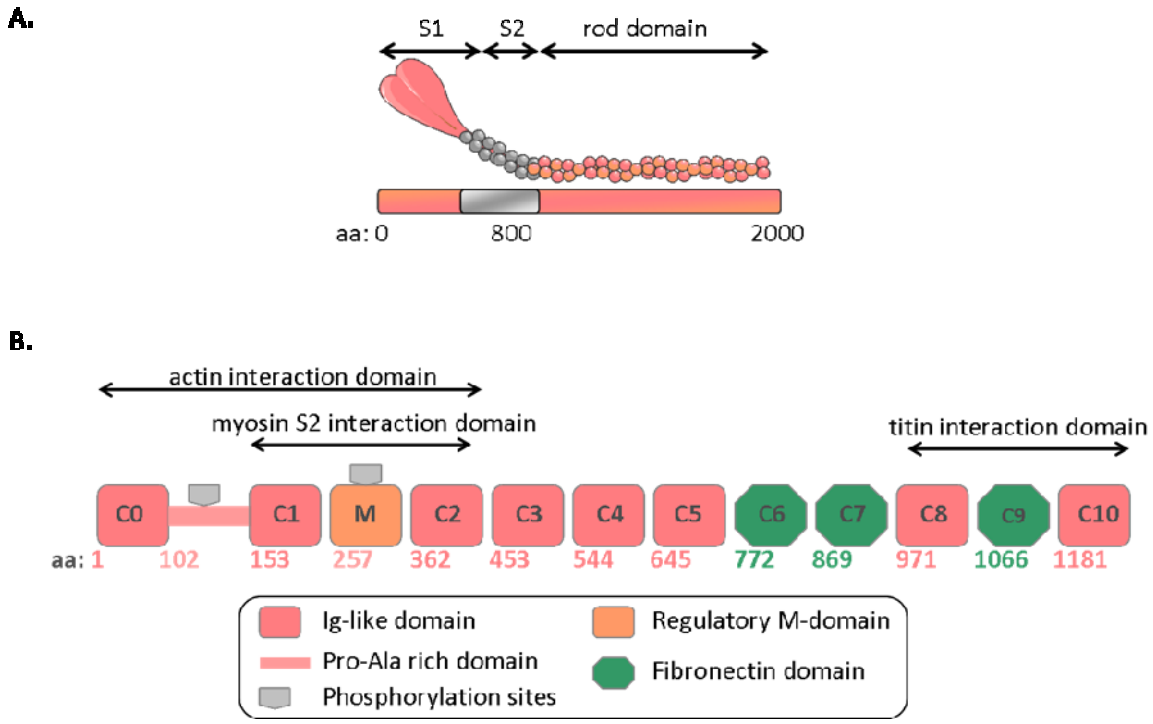

Figure 2 Schematic representations of the thick filament proteins in which most HCM mutations are found. **(A)** Cardiac myosin heavy chain **(B)** Cardiac myosin binding protein C.

Contractile proteins and sarcomere contraction

Cardiac muscle contraction is a calcium (Ca^{2+})-depending event in which all contractile proteins are involved. Contraction of the heart muscle, systole, is triggered by electrical activation (excitation) of the heart. Electrical stimulation (action potential) starts at the sinoatrial node in the right atrium and is propagated to the cardiomyocytes. During the depolarization phase of the action potential Ca^{2+} enters the cardiomyocyte via L-type Ca^{2+} channels. As a result the ryanodine receptors open, which leads to Ca^{2+} release from the sarcoplasmic reticulum (SR).[15] Subsequently, Ca^{2+} binds to cTnC. This signal is transmitted via cTnI and cTnT to cTm and actin, resulting in a conformational rearrangement in the cTn-cTm-actin complex, releasing the inhibitory action of cTnI on contraction. A cMyHC S1 binding site on actin is exposed, which enables myosin-actin interaction (Figure 1). The interaction of the cMyHC S1 domain with actin requires the hydrolysis of ATP by the myofibrillar ATPase and results in the power stroke. This occurs when adenosine diphosphate (ADP) and inorganic

phosphate (P_i) are released. P_i is the result of breakage of the high-energy phosphate bond of ATP by hydrolysis producing the energy needed for the power stroke.

$$ATP \rightarrow ADP + P_i + H^+ \quad (1)$$

This is the phase in which force is produced and the sarcomeres contract. Subsequently, ATP binds again to the S1 region leading to detachment of the myosin head from actin, which is the end of the cross-bridge cycle.[7] During diastole Ca^{2+} is taken up again into the SR by the SR Ca^{2+} ATPase (SERCA) and removed out of the cardiomyocyte via the Na^+/Ca^{2+} exchanger leading to cardiomyocyte relaxation.[15]

Role of thick and thin filament proteins in sarcomere contraction and relaxation

For proper sarcomere contraction cMyHC and actin need to interact directly, while all other sarcomere proteins have important regulatory roles in contraction and relaxation of the sarcomeres. These regulatory roles are influenced by post-translational modifications, such as phosphorylation. Phosphorylation is mediated by kinases. Protein kinase A (PKA) mediates the heart rate, contraction and relaxation via phosphorylation of, amongst others, cMyBP-C and cTnI.

cMyBP-C can be phosphorylated at more than 17 phosphorylation sites[16], but the relevance of several sites is currently not known. Phosphorylation of cMyBP-C at the PKA phosphorylation sites in the M-domain (Figure 2B) is important for healthy cardiac function as it is involved in cross-bridge kinetics. In its unphosphorylated form cMyBP-C interacts with the S2 domain of myosin by keeping the globular S1 heads more parallel to the thick filament backbone restraining them from binding with actin. Phosphorylation of cMyBPC weakens its interaction with the S2 domain of myosin, increasing cross-bridge cycling kinetics.[17]

The regulatory action of cTnI is controlled by PKA phosphorylation of mainly the sites Serine 23 and 24[18,19] and the recently identified Serine 149.[20] PKA-mediated Serine 23/24 phosphorylation decreases the Ca^{2+}-sensitivity of cTnC thereby reducing the probability of myosin to bind to actin. This favors cardiac relaxation.

cTnT affects the cross-bridge cycle by holding the entire cTn complex together as it interacts with cTnC-cTnI-cTm. Its main role is to modulate the position of cTm during the cross-bridge cycle. This interaction is associated with the inhibitory function of cTnI in the absence of Ca^{2+} and release of the inhibitory action of cTnI when Ca^{2+} is present.[21,22]

Modulation of cTm by cTnT and the resulting sliding movement on actin to release a cMyHC S1 binding site requires flexibility of the extended cTm molecule[7]. cTm is known to be a semi-flexible molecule. The flexibility might be due to an unstable, hydrophobic region in the middle of the cTm molecule. This region controls the function of cTm in its regulation of muscle activity.[23]

Mutations in genes encoding thick filament proteins and familial HCM

Over 80% of HCM disease-associated mutations reside in the genes *MYH7* and *MYBPC3*, which encode the thick filament proteins β-MyHC and cMyBP-C. Mutations in the genes

encoding thin filament proteins *TNNT2* (cTnT), *TNNI3* (cTnI), *TNNC1* (cTnC) and *TPM1* (cTM) have a prevalence of approximately 10%.[24,25] Until today 30-40% of all discovered HCM mutations are located in *MYH7*. Disease severity varies between families with different *MYH7* mutations, ranging from early disease onset and high incidence of sudden cardiac death or a less severe progression and outcome.[26] *MYH7* mutations are found at different locations of the protein ranging from the S1 domain to the rod domain (Figure 2A). Mutation location may underlie differences in functional consequences of the mutant protein and might explain the variability observed in clinical phenotype. In 1990 Geisterfer-Lowrance and co-workers[27] identified the first HCM-associated mutation in the actin-binding domain of β-MyHC. This was the arginine to glutamine transition at amino acid position 403, R403Q. Many studies have been performed to unravel the functional consequences of this mutation during the past 2 decades. The influence on cross-bridge kinetics has been extensively studied using a variety of experimental models and human tissue harboring the R403Q mutation. The effects were either increased or decreased cross-bridge kinetics depending on the model and/or method.[28] Despite many years of research, the functional consequences of this single mutation on cardiac contraction have not been entirely clarified yet. Moreover, limited information is available about the functional impact of other *MYH7* mutations.

Up to almost 200 HCM-associated mutations in *MYBPC3* have been reported after the first discovery of mutations in this gene.[29,30] Frequently, these mutations lead to truncated proteins, which are however not incorporated in the sarcomere.[31,32] Although the truncated protein was not observed in cardiac tissue of patients with *MYBPC3* truncating mutations, the level of full length cMyBP-C was significantly lower compared to non-failing donor hearts. This suggests that the *MYBPC3* truncating mutations result in haploinsufficiency (reduced protein level) rather than incorporation of a poison (truncated) peptide. Studies in rodents showed that cross-bridge kinetics was increased in the absence or at reduced levels of cMyBP-C.[33,34] Previous studies in single cardiomyocytes from HCM patients with founder *MYBPC3* truncating mutations revealed decreased maximal force generating capacity and increased myofilament Ca^{2+}-sensitivity.[31,35] The observed functional changes may be explained by secondary disease-related protein modifications such as altered phosphorylation. cMyBP-C phosphorylation was reduced in HCM patients without an identified sarcomeric protein mutation. However, in case of HCM with the founder mutations in *MYBPC3*, phosphorylation was not altered compared to controls.[36] This may be explained by cMyBP-C haploinsufficiency as the stoichiometry of activated kinases and the cMyBP-C protein levels might be altered in parallel, i.e. the reduction in protein level may be equal to the reduction in kinase activities in HCM with *MYBPC3* mutations.[36] Although many studies have been performed on effects of *MYBPC3* mutations in mice and human myocardium, the direct defects caused by *MYBPC3* mutations remain to be established.

Mutations in genes encoding thin filament proteins and familial HCM

cTnT was the first thin filament protein that was associated with HCM[37] and since then more than 30 *TNNT2* mutations have been discovered. A lot of these mutations are found at both the cTnC-cTnI and the cTm interaction sites. This implies that the mutant forms of cTnT alter contraction regulation due to their interaction with other thin filament proteins.[38,39] Mutations in *TNNT2* are especially known for their ability to increase myofilament Ca^{2+}-

sensitivity[40–43] and to increase susceptibility for arrhythmias.[44] Recently, it has been found in an *in vitro* study using recombinant troponin with *TNNT2* mutations that the conformational closure of the thin filament, i.e. impairment of Ca^{2+} to bind to cTnC, slowed down in case of *TNNT2* mutations, explaining the increased Ca^{2+}-sensitivity.[42]

HCM-causing mutations in *TNNI3* were first reported in 1997,[45] while the first mutation in *TNNC1* was found in 2001.[46] In general, HCM mutations in *TNNI3* and *TNNC1* have been associated with an increase in Ca^{2+}-sensitivity and decrease of cTnI phosphorylation.[47–49]

The first mutations in *TPM1* have been identified in 1994.[50] *TPM1* mutations are known to increase myofilament Ca^{2+}-sensitivity[51,52] and increase cTm flexibility.[53,54] The increased flexibility results in better accessibility of cMyHC-S1 binding sites on actin, which might underlie the increase in Ca^{2+}-sensitivity.[55]

HCM genotype versus phenotype

The functional mutation-related consequences found in various HCM disease models, including human HCM, are diverse. HCM mutations often result in decreased contractile protein phosphorylation and higher myofilament Ca^{2+}-sensitivity. An increase in myofilament Ca^{2+}-sensitivity may augment contractility though it may impair relaxation. A higher myofilament Ca^{2+}-sensitivity could also alter the Ca^{2+} transient by enhanced Ca^{2+} buffering at the sarcomeres leading to activation of Ca^{2+}-mediated pro-hypertrophic pathways.[15] In contrast to the increased myofilament Ca^{2+}-sensitivity, maximal force generating capacity was found to be decreased in human HCM tissue at the cellular level in human HCM samples with *MYBPC3* and *MYH7* mutations.[31,35,36] Moreover, as mentioned above, apart from myocardial dysfunction, LV remodeling is an important characteristic of the HCM phenotype. Hence, changes in myocardial function may be a primary consequence of the mutant proteins and/or may be due to secondary disease-related cardiac remodeling processes. A role for secondary disease-related factors was evident from studies in HCM patients without an identified pathological mutation (so-called sarcomere mutation-negative HCM), which show similar changes in post-translational modifications as observed in sarcomere mutation-positive patients.[36] The complex HCM phenotype makes it difficult to establish the direct link between the mutation and HCM disease symptoms. In **the first part of this thesis** we aimed to discriminate between the direct consequences of the mutation and the disease-related morphological changes combining functional and histological analyses in single cardiomyocytes and single myofibrils from patients with mutations in genes encoding thick and thin filament proteins (Chapters 2&3). The relevance of our *in vitro* measurements in single cardiomyocytes for *in vivo* cardiac performance was assessed by combining *in vitro* (cardiac tissue) with in vivo cardiac function (echocardiography) measurements in the same patients (Chapter 4). Through our (inter)national collaborations we were in the unique position to study a large number of cardiac samples from patients with mutations in *MYBPC3*, *MYH7*, *TPM1*, *TNNI3* or *TNNT2*. A variety of different mutations were studied in relation to force generating capacity and cellular morphology.

Energetic status of the (HCM) heart

As mentioned, ATP is hydrolyzed (equation 1) by the actin-binding domain of the thick filament protein myosin to produce the energy for the power stroke. The predominant amount of ATP is synthesized by oxidative phosphorylation of fatty acids in the mitochondria and phosphotransferase reactions catalyzed by creatine kinase under healthy conditions. When ATP demand exceeds ATP availability during stress conditions other pathways are activated such as glycolysis and de novo pathways.[56]

ATP requiring processes will be impaired, such as the actin-myosin interaction, when the ATP hydrolysis products ADP and P_i are accumulating, which means that there is a lack of (Gibbs) free energy or phosphorylation potential (equation 2), to fuel the ATP-requiring reactions.

Phosphorylation potential = $[ATP]/[ADP][P_i]$ (2)

The concentrations in healthy myocardium are [ATP]: 10 mmol/L; [ADP]: <50 micromol/L (metabolically active ADP pool); $[P_i]$: < 1 mmol/L and a phosphorylation potential of>200 $mmol/L^{-1}$. Since the turnover of ATP by hydrolysis is higher than the described amount of intracellular ATP, it is very important to maintain a high ATP supply, i.e. phosphorylation potential, to meet cardiac demands.[57] Thereto the heart uses an energy reserve system. The primary energy reserve is the creatine kinase (CK) system (equation 3). CK is abundant in the heart.

$PCr + ADP + H^+ <-> creatine + ATP$ (catalyzed by CK) (3)

When the amount of creatine in the heart is lower, the total amount of phosphocreatine (PCr) should be lower as well, leading to a decrease in the PCr/ATP ratio. This decreases the phosphorylation potential (equation 2), thereby decreasing the energetic yield as ATP hydrolysis is impaired.[58,59] Over the years, this ratio has become an important predictor of the energetic state of the heart and cardiovascular mortality.[60] ^{31}P NMR studies in patients with dilated cardiomyopathy and in patients with heart failure[61,62] showed a decrease in PCr/ATP. Interestingly, a decrease in this ratio has been found in non-failing human HCM patients with various sarcomeric gene mutations as well.[63] The decrease in PCR/ATP ratio has been proposed as possible common denominator in HCM development, i.e. energetic compromise of the heart. However, this ratio does not indicate what happens at the sarcomere level with respect to myofibrillar ATPase activity. Studies in rodent HCM models with mutations in *TNNT2* showed an increase in energetic cost of tension generation at the level of the sarcomere.[64–66] Moreover, recent *in vitro* motility and ATPase assays and single myofibril studies in human HCM with *MYH7* mutations suggested a decrease in cross-bridge efficiency as well.[67,68] However, energetic defects at the level of the sarcomere, as a cause of inefficient cardiac performance, have not been proven in human HCM. The **second part of this thesis** therefore focuses on the impact of mutations in thick and thin filament proteins on myocardial energetics in human HCM (Chapters 5, 6&7). Also here, we step from *in vitro* measurements of myocardial energetics to assessment of *in vivo* myocardial efficiency in individuals carrying HCM mutations (Chapter 8).

Aim of this thesis

The mechanisms regarding the genotype-phenotype relation in human HCM have not yet been elucidated. The aim of this thesis is to distinguish cellular morphological changes from mutation-induced intrinsic sarcomere defects in human HCM with various sarcomeric gene mutations. Our studies provide insight in the contractile and energetic phenotype at the level of the sarcomere. Moreover, we translate our *in vitro* studies in cardiac muscle samples to *in vivo* cardiac performance assessed in the clinical setting to establish the relevance of the cellular changes for *in vivo* cardiac performance.

Thesis outline

Part 1 Cellular remodeling and sarcomere force generating capacity in human hypertrophic cardiomyopathy

To study the effect of the heterozygous R723G *MYH7* mutation we investigated in Chapter 2 contractile function of membrane-permeabilized single LV cardiomyocytes and the *Musculus soleus* fibers of HCM patients harboring this mutation. In addition, myofilament protein phosphorylation was determined using the Pro-Q Diamond/Sypro Ruby staining gels and Western Blot analysis. Cellular remodeling was analyzed by means of histochemistry and electron microscopy. LV tissue of non-failing donors was used as control.

In Chapter 3 we further elaborated on the influence of cellular remodeling on contractile function in HCM tissue of patients with mutations in *MYBPC3*, *MYH7*, *TPM1*, *TNNI3* and *TNNT2* and in tissue of sarcomere mutation-negative HCM patients. Maximal force generating capacity was measured in membrane-permeabilized single cardiomyocytes and single myofibrils. Moreover, cross-sectional area, fibrosis and myofibrillar density was analyzed using histochemistry and electron microscopy. LV tissue of non-failing donors and of patients with LV remodeling due to aortic stenosis were used as control.

Chapter 4 connects *in vitro* patient data with *in vivo* cardiac performance of the same patients. Segmental systolic strain and strain rate were analyzed using speckle tracking echocardiography in HCM sarcomere mutation-positive and sarcomere mutation-negative patients. In addition, maximal force generating capacity was measured in membrane-permeabilized single cardiomyocytes isolated from IVS tissue of these patients obtained during myectomy surgery.

Part 2 Myocardial energy utilization in human hypertrophic cardiomyopathy

In Chapter 5 we studied whether cross-bridge detachment rates correlate with the energetic cost of tension generation at the level of the sarcomere in human HCM with the heterozygous R403Q *MYH7* mutation. Cross-bridge kinetics was analyzed in membrane-permeabilized cardiac myofibrils and energetic cost of tension generation in membrane-permeabilized multicellular cardiac muscle strips of the same patients. HCM tissue of sarcomere mutation-negative patients was used as a reference group.

Chapter 6 focuses on the effects of the homozygous K280N *TNNT2* mutation on cross-bridge kinetics measured in membrane-permeabilized cardiac myofibrils, and sarcomere energetics measured in membrane-permeabilized multicellular cardiac muscle strips of a patient with this mutation. HCM tissue of sarcomere mutation-negative patients and LV tissue of patients with LV remodeling due to aortic stenosis were used as reference groups. To investigate to what extent the incorporation of this mutation directly causes changes in contractile function, the mutated cTn complex in the K280N mutated preparations was exchanged with wild-type cTn complex and endogenous cTn was exchanged with recombinant K280N cTn in control tissue. The amount of exchange was determined using Pro-Q Diamond/Sypro Ruby stained gels and Western blot analysis.

In Chapter 7 we describe the influence of sarcomeric gene mutation location and LV remodeling on the energetic cost of sarcomere contraction. Sarcomere energetics was measured in membrane-permeabilized multicellular cardiac muscle strips of patients with *MYBPC3* and *MYH7* mutations located in different regions of these genes; multicellular muscle strips of sarcomere mutation-negative HCM patients and of patients with LV remodeling due to aortic stenosis served as controls.

Chapter 8 connects *in vitro* patient data with *in vivo* patient data. Next to studying energetic cost of sarcomere contraction in manifest HCM patients, we investigated whether myocardial energetics would be affected in the pre-hypertrophic disease state as well. Myocardial external efficiency was analyzed in pre-hypertrophic individuals carrying a mutation in *MYBPC3* and *MYH7* and in healthy volunteers by means of positron emission tomography and cardiac magnetic resonance imaging. Moreover, sarcomere energetics was analyzed in multicellular cardiac muscle strips of manifest patients harboring mutations in *MYBPC3* and *MYH7*. Tissue of sarcomere mutation-negative HCM patients was used as a reference group.

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