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Summary, Conclusions & Future Perspectives

Familial hypertrophic cardiomyopathy: an energetic story about cellular remodeling and sarcomere function.

The purpose of this thesis was to obtain better insight in the complex genotype-phenotype relation present in human hypertrophic cardiomyopathy (HCM). We distinguished cellular morphological changes from HCM mutation-induced intrinsic effects on the contractile and energetic phenotype of the sarcomere. *In vitro* studies in human cardiac muscle tissue were translated to *in vivo* human cardiac performance to assess the relevance of cellular morphological and contractile changes for *in vivo* cardiac performance. The main findings of this thesis are summarized below and in Tables 1 and 2.

Mutation versus cellular remodeling

Cellular remodeling in HCM might be an important cause of cardiomyocyte and overall cardiac dysfunction. It could mask the mutation-induced functional changes of the affected sarcomeres. In Chapter 2 we investigated cardiomyocyte contractile function and cellular remodeling of HCM patients with the heterozygous R723G *MYH7* mutation compared with non-failing donor cardiomyocytes. Moreover, contractile function was analyzed in slow fibers of the *M. soleus* of one of the HCM patients to investigate differences between mutation-induced effects in skeletal and cardiac muscle. In single cardiomyocytes from $MyHC_{R723G}$, maximal force generating capacity was significantly lower compared to donor cardiomyocytes. However, in the slow skeletal fibers maximal force generating capacity (F_{max}) was significantly higher than in controls. There were no differences in myofilament Ca^{2+}-senstivity between the $MyHC_{R723G}$ and donor cardiomyocytes. After PKA incubation, however, Ca^{2+}-sensitivity was lower in the $MyHC_{R723G}$ than in donor cardiomyocytes and force remained reduced in $MyHC_{R723G}$. Ca^{2+}-sensitivity was also lower in *M. soleus* from the HCM patient compared to controls. Phosphorylation of troponin I, troponin T, myosin binding protein-C and myosin light chain 2 was significantly lower in $MyHC_{R723G}$ hearts compared to donor myocardium. Interestingly, $MyHC_{R723G}$ cardiomyocytes showed myofibrillar disarray and myofibrillar density was lower compared with donor myocardium. The $MyHC_{R723G}$ mutation itself reduces Ca^{2+}-sensitivity in both cardiomyocytes and skeletal slow fibers, while reduced phosphorylation appears to compensate for the reduced myofilament Ca^{2+}-sensitivity in cardiomyocytes. Cardiomyocyte F_{max} does not depend on phosphorylation directly, but seems to be the resultant of a lower myofibrillar density and myofibrillar disarray in cardiac tissue with the $MyHC_{R723G}$ mutation.

Based on the previous found reduction in myofibrillar density in HCM due to the R723G mutation in Chapter 2, the next step was to discern the influence of cellular remodeling from mutation-induced sarcomeric defects on maximal force generating capacity in tissue of manifest human HCM patients. Therefore, in Chapter 3 the myofibrillar density and cardiomyocyte area was compared of single cardiomyocytes harboring mutations in thick filament (*MYBPC3*, *MYH7*) and thin filament (*TPM1*, *TNNI3* and *TNNT2*) proteins with single cardiomyocytes of sarcomere mutation-negative (HCM_{smn}) patients, patients with left ventricular (LV) hypertrophy due to aortic stenosis (LVH_{ao}) and non-failing donors. In

addition, the amount of fibrosis was analyzed. Moreover, F_{max} was investigated in single cardiomyocytes and myofibrils from sarcomere mutation-positive patients in comparison with HCM_{smn}, LVH_{ao} and non-failing donors. Although cardiomyocyte area (CSA) was significantly higher in all HCM cardiomyocytes compared to donor cells, it was found that CSA was even larger in cardiomyocytes harboring a sarcomere mutation compared to HCM_{smn}. Myofibrillar density was decreased in all HCM and LVH_{ao} cardiomyocytes compared to donor cardiomyocytes, however the decrease was largest in cardiomyocytes from sarcomere mutation-positive HCM patients. There was a negative correlation between myofibrillar density and cardiomyocyte hypertrophy. F_{max} of all HCM single cardiomyocytes, but profoundly in *MYH7*$_{mut}$ cells, was decreased compared to donor cardiomyocytes. Interestingly, F_{max} in single *MYH7*$_{mut}$ cardiomyocytes was not restored to donor values after correction for myofibrillar density, while the reduction in myofibrillar density could explain the lower F_{max} in cells with a *MYBPC3* mutation. The amount of fibrosis was increased in HCM mutation-positive and HCM mutation-negative tissue however did not correlate with F_{max}.

The reduction in F_{max} due to *MYH7* mutations was confirmed by the force measurements in single myofibrils harboring *MYH7* mutations. Therefore, the conclusion is that the decrease in maximal force generating capacity in HCM patients is mainly due to the cellular hypertrophy and reduced myofibrillar density. The lower F_{max} in *MYH7*$_{mut}$ tissue, however, is directly caused by the presence of the mutation suggesting hypocontractile sarcomeres as primary abnormality in patients with *MYH7* mutations.

Regional versus global contractility

The fact that HCM mutations (directly or indirectly) result in a decrease in force generating capacity of individual cardiomyocytes (Chapters 2&3) is interesting from a clinical point of view as HCM patients often show no changes in global systolic function. Therefore, in Chapter 4 *in vitro* cell measurements was combined with *in vivo* measurements of regional contractile function. F_{max} was measured in single cardiomyocytes of the same HCM patients harboring various gene mutations in which regional systolic strain was analyzed with speckle tracking echocardiography. Interestingly, the significant decrease in F_{max} in single cardiomyocytes correlated with a decrease in systolic strain at regional level. Despite lack of global systolic dysfunction generally in HCM patients, the results show that the hypocontractile cardiomyocytes underlie regional systolic impairment, which might be caused by the sarcomeric gene mutations. Table 1 shows the main results of **part 1** of this thesis.

Table 1. Effects of sarcomere mutations on remodeling and contractility

	F_{max}	Myofibrillar density	F_{max} corrected for myofibrillar density	CSA	Fibrosis	Regional systolic strain
LVH_{ao}	↓	↓↓	=	↑	=	n.a.
HCM_{smn}	↓	↓	=	↑	↑	↓
***MYBPC3*$_{mut}$**	↓	↓↓	=	↑↑	↑	↓
***MYH7*$_{mut}$**	↓↓	↓↓	↓	↑↑	↑	↓
***TMP1*$_{mut}$**	↓↓	↓↓	↓	↑↑	↑	↓
***TNNI3*$_{mut}$**	↓	↓↓	=	↑↑	↑	↓
***TNNT2*$_{mut}$**	↓	↓↓	↓	↑↑	↑	n.a.

The main findings presented regarding remodeling and contractility *in vitro* using human cardiac HCM tissue (F_{max}, myofibrillar density, CSA and fibrosis) and *in vivo* using HCM patients (regional systolic strain). The 'arrows' and 'equals to' (=) sign indicate the effect compared with non-failing donor tissue and healthy subjects. A double arrow represents an even larger effect as the parameter was altered compared with HCM_{smn} as well. N.a.; not analyzed.

Disturbed energetics

As the PCr/ATP ratio, a measure of the energetic status of the heart, is an important factor in heart failure development it has been proposed as disease modifier in HCM as well. Previous studies in both animal models and overt human HCM patients harboring various sarcomeric gene mutations revealed a decrease in the PCr/ATP ratio, suggesting a deficiency in the energetic status of the heart. In addition, studies in human HCM tissue with the first identified HCM-associated mutation, the R403Q *MYH7* mutation, suggested a decrease in efficiency based on faster cross-bridge kinetics. In Chapter 5 we investigated whether faster cross-bridge relaxation kinetics indeed relates to a higher energetic cost of sarcomeric force development, i.e. tension cost, in human HCM tissue harboring this mutation. The R403Q mutation is particularly interesting as it is located in the globular head of myosin, which is responsible for the interaction with actin. A mutation in this area is very likely to interfere with the motor function of myosin. Cross-bridge relaxation kinetics was analyzed in single myofibril preparations of 3 $MyHC_{R403Q}$ patients and TC in multicellular muscle strips of the same patients. TC is the ratio between ATPase activity and force generating capacity normalized to cross-sectional area (tension). Preparations of 9 HCM_{smn} were used as controls. Cross-bridge slow relaxation kinetics was significantly higher in $MyHC_{R403Q}$ myofibrils compared to HCM_{smn} and TC was significantly higher in the $MyHC_{R403Q}$ muscle strips as well. As the R403Q mutation is heterozygous, mRNA expression was analyzed. R403Q mRNA expression was on average 41% of total *MYH7* mRNA and did not correlate with any of the functional parameters. However, a clear positive linear correlation appeared between the slow relaxation kinetics and TC from which we can indeed conclude that faster cross-bridge relaxation kinetics results in an increase in energetic cost of tension generation in human HCM with the R403Q mutation.

The R403Q mutation was a heterozygous mutation resulting in a healthy and diseased allele. However, in Chapter 6 cross-bridge kinetics and sarcomere energetics were studied in a unique patient sample with a homozygous mutation in *TNNT2*; K280N. This mutation results in a 100% expression of mutant protein. The increase in cross-bridge detachment rate observed in single myofibrils of this patient suggested an increase in energetic cost of contraction. Indeed, a higher TC was found in the multicellular cardiac

muscle strips of this patient compared to HCM_{smn} preparations. Moreover, exchanging endogenous $cTnT_{K280N}$ with wild-type cTn in both single myofibrils and muscle strips of this patient slowed down kinetics and lowered TC, confirming a clear causal relation between the mutation and the observed functional defects.

Chapter 7 investigated the effect of mutation location in the *MYBPC3* and *MYH7* genes on TC. In addition, the possible influence of LV remodeling was taken into account. To accomplish this, TC was not only measured in muscle strips of 16 *MYBPC3* and 11 *MYH7* patients, but also in 11 HCM_{smn} patients and in muscle strips of 12 patients with LV remodeling due to aortic stenosis. TC was significantly higher in both mutation groups compared to HCM_{smn} indicating that energetics of contraction is impaired by the presence of a sarcomere mutation per se. Mutations in the C5-C7 domains of cMyBP-C resulted in higher tensin cost compared with mutations in other domains. Mutations in the S1 domain of cMyHC showed the highest increase in TC compared with the S2 and rod domains. This suggests that mutation location is an important determinant regarding changes in sarcomere energetic cost of contraction. In addition, a similar increase in TC was observed in LVH_{ao} muscle strips as in the HCM mutation groups relative to the HCM_{smn} group, however remodeling (higher interventricular septum thickness) was more severe in the HCM patient groups, Therefore, in addition to the effect of remodeling on energetic cost of contraction in the HCM and LVH_{ao} groups, another mechanism might underlie the TC changes at sarcomere level in LVH due to a secondary cause compared with HCM.

As an increase in energetic cost of tension generation at the cellular level was observed, the next question was whether an energetic deficit is already visible at an early stage of HCM. In Chapter 8 we therefore combined the *in vitro* TC measurements in muscle strips of 21 manifest HCM patients with *MYBPC3* and *MYH7* mutations and 6 HCM_{smn} patients with *in vivo* analyses of myocardial external efficiency in **pre**-hypertrophic mutation carriers. For the *in vivo* analyses healthy volunteers were used as controls. Myocardial external efficiency (MEE) is the ratio between myocardial external work, analyzed with cardiac magnetic resonance (CMR) imaging and myocardial oxygen consumption, investigated with positron emission tomography (PET). MEE was significantly lower in both mutation carrier groups compared to controls. Moreover, manifest $MYH7_{mut}$ patients and *MYH7* mutation carriers revealed a higher TC and lower MEE compared to manifest $MYBPC3_{mut}$ patients and *MYBPC3* mutation carriers, respectively. Based on these results it can be concluded that changes in myocardial energetic cost of contraction are not only gene-specific, but also visible at an early disease stage. Evidence is provided in human HCM that an energetic deficit may be a target of metabolic treatment even at the pre-hypertrophic stage of HCM. Table 2 summarizes the main results of **part 2** of this thesis.

Table 2. Effects of sarcomere mutations on sarcomere kinetics, energetics and myocardial performance.

	Slow k_{rel}	Slow k_{rel} after exchange	F_{max}	ATPase activity	TC	TC after exchange	EW	MVO_2	MEE
LVH_{ao}	=	n.a.	=	↑	↑	n.a.	n.a.	n.a	n.a
***MYBPC3*$_{mut}$ (multiple patients)**	n.a.	n.a.	↓	=	↑	n.a.	↓	=	↓
***MYH7*$_{mut}$ (multiple patients)**	n.a.	n.a.	↓↓	↓↓	↑↑	n.a.	↓	=	↓↓
$MyHC_{R403Q}$	↑	n.a.	↓	↓	↑	n.a.	n.a	n.a	n.a
MyHC (S1) (multiple patients)	n.a.	n.a.	↓	↓	↑	n.a.	n.a	n.a	n.a
MyHC (S2/rod) (multiple patients)	n.a.	n.a.	=↓	=↓	=↑	n.a.	n.a	n.a	n.a
$cTnT_{K280N}$	↑	↓	↓	↓	↑	↓	n.a	n.a	n.a

The main findings presented in HCM tissue with sarcomeric gene mutations (slow k_{rel}, F_{max}, ATPase activity, TC) and pre-hypertrophic mutation carriers (EW, MVO_2 and MEE). TC; TC, EW; myocardial external work, MVO_2; myocardial oxygen consumption, MEE; myocardial external efficiency and n.a; not analyzed. The 'arrows' and 'equals to' (=) signs represent comparisons with HCM_{smn} tissue and healthy subjects. A double arrow represents an even larger effect as the parameter was altered compared with *MYBPC3*$_{mut}$ as well.

Conclusions

The decrease in maximal force generating capacity found in HCM cardiomyocytes harboring various sarcomeric gene mutations is mostly due to cellular remodeling, i.e. cellular hypertrophy and reduced myofibrillar density. However, incorporation of mutant MyHC seems to directly contribute to the reduction in force generating capacity by causing hypocontractile sarcomeres (Chapters 2&3). Moreover, the reduction in cellular force generating capacity correlated with regional systolic strain. Overall, these data reveal systolic dysfunction at a regional level, which may represent a trigger of HCM development in particular in individuals carrying a *MYH7* mutation (Chapter 4).

HCM mutations do not only affect force generating capacity, but cross-bridge relaxation kinetics and energetics as well. The $MyHC_{R403Q}$ and $cTnT_{K280N}$ mutations increase slow relaxation kinetics of affected cross-bridges, which directly correlated with an increase in energetic cost of tension generation (Chapters 5&6). Changes in relaxation kinetics and TC in the $cTnT_{K280N}$ patient were rescued by exchanging the mutant $cTnT_{K280N}$ with WT cTnT (Chapter 6). Moreover, *MYBPC3* and *MYH7* mutations in general decrease the efficiency of myocardial contraction in both manifest HCM tissue as well as in pre-hypertrophic mutation carriers (Chapters 7, 8). The defects were largest in individuals harboring *MYH7* mutations, although there is a clear dependence on mutation location (Chapter 7). Overall our data suggest that metabolic treatment targeting efficiency of myocardial contraction might be beneficial for HCM patients, especially those harboring *MYH7* mutations (Chapters 7&8).

Future perspectives

HCM is a highly prevalent cardiovascular disease, affecting a large population of all ages. There is, however, no treatment available to prevent disease onset and cardiac death. This is possibly mainly related to the diversity of the involved molecular pathways regarding the origin of HCM disease. Eventually, a treatment target of which a large group of HCM patients might benefit is the ideal perspective. However, identification of a treatment target is challenging as based on this thesis we can state that although sarcomeric gene mutations are important causal factors, cardiomyocyte remodeling has been found to be a confounding factor as it masks intrinsic sarcomere defects. In addition, each gene or even a specific HCM mutation acts differently on sarcomere function. Moreover, affected gene and mutation location appear to be important determinants of functional changes, which warrant more studies in human samples, which highly depend on good collaborations between preclinical en clinical departments.

Mutation expression and sarcomere function

Next to the presence of a mutation and mutation location, it is of importance to elaborate on the expression level which is needed to perturb contractile function as we observe the highest TC in a sample with a homozygous $cTnT_{K280N}$ mutation resulting in 100% mutant protein expression (Chapter 6). The effect of this full expression of mutated protein proved clearly the negative effect on sarcomere function. The techniques used to exchange the $cTnT_{K280N}$ with the WT cTnT and vice versa enable to investigate how much of the $cTnT_{K280N}$ needs to be expressed to induce a diseased contractile phenotype.

The other studied mutations were heterozygous leading to a healthy and a diseased allele. However, heterozygous mutations do not lead to a 50% expression level of both alleles as proved previously in tissue harboring *MYH7* mutations.[1,2] An effect on contractile function would be expected due to this allelic imbalance as well. Global mRNA expression levels did show differences in expression among the patients, but there was no correlation with TC. Although mRNA expression provides insight in the protein levels as well, the mRNA levels remain snapshots in time. Therefore, a targeted antibody against a specific mutated protein, such as $cMyHC_{R403Q}$, would provide direct insight in mutated protein levels. Nevertheless, it is quite challenging to produce a specific antibody against a protein which has only a single amino acid change. In the future, mutant protein expression may be revealed by mass spectrometry.

There were not only differences in TC among different patients with the same mutation, but also among the individual preparations from one patient. In addition to global allelic imbalance, there is proof that allelic imbalance plays a role at individual cardiomyocyte level.[3] Further research in this field might contribute to explain the differences in energetic cost of tension generation in individual preparations of tissue from one patient with a specific sarcomeric gene mutation.

In case of the heterozygous *MYBPC3* mutations a similar approach would be of interest as most of these mutations lead to haploinsufficiency. The influence of in this case healthy protein expression on contractile function could be investigated by incubating

preparations, either single cardiomyocytes or multicellular muscle strips, with full length cMyBP-C in addition to the baseline measurements.

Vascular remodeling and myocardial energetics

We studied the influence of different HCM mutations on cardiomyocyte remodeling and sarcomere function. Recently more attention has been given to microvascular remodeling. Coronary microvascular dysfunction (CMD) might even be an important primary feature of HCM leading to LV cellular remodeling itself, myocardial ischemia and eventually sudden cardiac death.[4–6] A deficit in oxygen delivery implicates changes in energetic status of the heart. A study on changes in myocardial blood flow (MBF) between manifest HCM mutation-positive and mutation-negative patients revealed a lower MBF in the mutation-positive patient group using PET analysis. This reduction was again mostly evident in manifest HCM patients with *MYH7* mutations.[7] There is no proof of MBF alterations in *MYH7* mutation carriers in the pre-hypertrophic disease stage, although it is known not to be altered in case of *MYBPC3* mutation carriers.[8]

Nevertheless, as described in this thesis, we revealed that myocardial energetics was more severely affected not only in manifest *MYH7* HCM patients, but also in *MYH7* mutation carriers compared with *MYBPC3* mutation carriers. This indicates that the energetic defect is gene-dependent already at an early disease stage. Therefore, it would be of interest to find out whether CMD is present as well in a pre-hypertrophic disease stage in *MYH7* mutation carriers. The preposition that changes in myocardial energetics precede the other HCM hallmarks[8], including CMD, might be gene-dependent as well.

Future research should focus on treatment regarding these two important hallmarks of HCM disease. It is known that reducing the LVOT obstruction in manifest HCM patients improves microvascular dysfunction[9–11] and increases the efficiency in these patients.[9] However, treatment in the pre-hypertrophic stage of the disease should focus on energetics. Metabolic treatment, such as perhexiline responsible for shifting fatty acid oxidation towards the more efficient glucose metabolism[12] might already be beneficial in the early disease stage, especially in case of *MYH7* mutation carriers.

Models to study HCM disease

As HCM is thought to be a disease of the sarcomere it is of great importance that the proper "model" is used to study the molecular origin of the disease. In this thesis only human HCM tissue was studied. However, this is highly dependent on availability as myectomy surgery or transplantation surgery is required for tissue collection. To overcome human HCM tissue availability issues and the option to study the influence of a mutation from birth on, transgenic animal models are often thought to be crucial. A large number of studies have been performed in rodent disease models; transgenic rodents harboring HCM mutations. However, one should keep in mind, especially with respect to models harboring myosin mutations, that rodents mostly express α-myosin heavy chain (*MYH6*) in the LV, while human LVs express mostly β-myosin heavy chain (*MYH7*).[13] From a kinetic point of view these two isoforms have distinct properties as the α-myosin heavy chain is more efficient and faster compared with the β-myosin heavy chain.[14] Options are replacing the endogenous α-myosin heavy chain in rodents for β-myosin heavy chain[14] or usage of larger animals

expressing β-myosin heavy chain similar as in human such as a rabbit model harboring the cMyHC R403Q mutation.[15,16] In the future a transgenic porcine model may represent a proper human-like model to study disease progression.

Another interesting option is based on engineered heart tissue (EHT). An EHT model has been developed based on neonatal cardiac mouse cells harboring a HCM *MYBPC3* mutation. Data regarding contractile function and drug responses are already available.[17] The next step would be the implementation of a HCM mutation in human EHT from human induced pluripotent stem cells.[18,19] This enables a new field of research; testing the influence of a variety of HCM mutations in a human-like environment on cellular morphology and contractile function.

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