SUMMARY, CONCLUSIONS & FUTURE PERSPECTIVES

"All truths are easy to understand once they are discovered; the point is to discover them."

Galileo Galilei

SUMMARY

The purpose of this thesis was to dissect the cellular and mechanical alterations related to myocardial inactivation that limit diastolic performance in patients with familial hypertrophic cardiomyopathy (HCM) and patients with non-familial causes of heart failure. From experiments at the cellular and whole organ level, in humans and rats, we are able to translate our findings into the *in vivo* human setting to obtain a better understanding of human cardiac performance.

High myofilament Ca^{2+}-sensitivity is a common characteristic in human HCM

Significant efforts have been made to identify a common pathomechanism triggered by mutations that could explain the multitude of intracellular events observed in HCM. Impaired relaxation is a general finding in humans and animals carrying HCM gene mutations. Impaired diastolic function is observed before the development of hypertrophy and fibrosis. Impaired relaxation may be explained by the elevated myofilament Ca^{2+}-sensitivity (the "high Ca^{2+}-state"), which is observed in the majority of *in vitro* and transgenic mice studies. The high myofilament Ca^{2+}-sensitivity would be sufficient to cause 'systolic' activation at low [Ca^{2+}] capable of delaying the onset of relaxation. In Chapter 3 we performed a comprehensive study on a large set of human HCM samples to identify if high myofilament Ca^{2+}-sensitivity is characteristic of human HCM with mutations in thick- and thin-filament proteins. Since high myofilament Ca^{2+} sensitization depends on muscle length and the phosphorylation state of myofilaments, cardiomyocyte force measurements were performed at two different sarcomere lengths (i.e. length-dependent activation), without and with treatment with exogenous protein kinase A (PKA). Measurements were performed in HCM samples and compared with sarcomere mutation-negative and non-failing donors. We observed that high myofilament Ca^{2+}-sensitivity is a common characteristic of human HCM, though partly reflects the hypophosphorylated state of PKA targets and cannot solely be related to the mutant protein itself. In addition, reduced length-dependent myofilament Ca^{2+}-activation was shown to represent a common pathomechanism in HCM with missense mutations, which is independent of the phosphorylation background. Moreover, a blunted increase in maximal force development upon an increase in sarcomere length was found in HCM carrying missense mutations. The diminished length-dependent myofilament Ca^{2+}-activation was not rescued upon PKA administration in HCM with sarcomeric mutations. Finally, we provided direct proof that mutant troponin T (at least for the K280N) impairs length-dependent activation, supporting that mutations affect thin-filament geometry.

High myofilament Ca^{2+}-sensitivity and length-dependent activation are regulated by PKA and PKC phosphorylation of cardiac troponin I

In Chapter 4 the effects of troponin I (cTnI) phosphorylation at threonine 143 (Thr143) site by protein kinase C (PKC) on myofilament Ca^{2+}-sensitivity and length-dependent activation were studied. Because Thr143 is a well characterized PKC-phosphorylation site that is highly phosphorylated in human failing hearts, we investigated if phosphorylation of Thr143 modifies myofilament Ca^{2+}-sensitivity and length-dependent activation in human cardiomyocytes. PKC-mediated phosphorylation at Thr143 may be detrimental for the diastolic phase. The effects of Thr143 phosphorylation were compared with the well-known protein kinase A (PKA) phosphosites serines 23 and 24 (Ser23/24) of cTnI. Troponin exchange experiments were performed in membrane-permeabilized human cardiomyocytes. Isometric force was measured at various [Ca^{2+}] in exchanged cardiomyocytes with recombinant wild-type (Wt) troponin or troponin mutated at the PKC site Thr143, or Ser23/24 into aspartic acid (D) or alanine (A) to mimic phosphorylation and dephosphorylation, respectively. In troponin-exchanged donor cardiomyocytes experiments were repeated after incubation with exogenous PKA. Pseudo-phosphorylation of Thr143 site increased Ca^{2+}-sensitivity compared to controls (Wt) without affecting length-dependent activation of control cardiomyocytes. Subsequent PKA treatment enhanced the length-dependent shift in Ca^{2+}-sensitivity after Wt and 143D exchange. Exchange with Ser23/24 phosphosites demonstrated that pseudo-phosphorylation of both Ser23 and Ser24 sites is required for the length-dependent increase in Ca^{2+}-sensitivity. cTnI pseudo-phosphorylation did not alter length-dependent changes in maximal force. In conclusion, phosphorylation at Thr143 enhances myofilament Ca^{2+}-sensitivity without affecting length-dependent activation, while Ser23/24 bisphosphorylation is needed to enhance the length-dependent increase in myofilament Ca^{2+}-sensitivity. This study provides evidence that low PKA-mediated phosphorylation as observed in HCM cardiomyopathy may cause the high Ca^{2+}-sensitivity (the "high Ca^{2+}-state") and impair length-dependent activation

Energy depletion and diastolic dysfunction - detrimental effects of high ADP

On the basis of the previous findings that human HCM tissue confers a high degree of thin-filament activation, even at low Ca^{2+}, we sought to characterize the impact of sustained basal systolic stress in relation to myocardial energy deficiency. Increased myofilament ATP consumption has been observed in HCM animal models and our group recently confirmed this in human HCM patients, evident from the increased cost (ATP utilization) for myofilament contraction, i.e. reduced tension cost. The impaired tension cost may result from an increased Ca^{2+}-induced myosin-ATPase activity, which would decrease myocardial energy supply for other ATP-dependent processes in the cardiac muscle cells. There is a general consensus that the myocardial energy reservoir in HCM patients is reduced, as a result of reductions in ATP content, also termed the "myocardial energy depletion hypothesis". Although attractive, this hypothesis fails to address the paradigm that *in vivo* ATP levels at rest in animal models

of heart failure are mostly unchanged and that even during increased workloads, the [ATP] is never rate-limiting to power actomyosin interactions. To this end, in Chapter 5 we performed a basal study ranging from experiments at the cellular level to the whole organ, in humans and rats, as to provide the proof-of-concept that myocardial accumulation of ADP (instead of reduced ATP), even in the micromolar amount, can contribute to diastolic dysfunction. We provide evidence that synergistic actions of physiological levels of ADP and diastolic Ca^{2+} increase actomyosin interactions, which elevate myocardial stiffness and limit proper filling of the heart. Our findings show that increased cross-bridge interactions may lead to diastolic dysfunction in environments with elevated ADP and diastolic Ca^{2+}, evidenced by high cardiomyocyte stiffness and impaired diastolic re-lengthening, associated with limited ventricular compliance. In addition, we also showed that the contribution of cross-bridge interaction to total diastolic stress in rat membrane-permeabilized cardiomyocytes is much higher than previously considered. Perhaps most importantly, we showed that physiological levels of ADP (which increase strong-binding cross-bridge formation) are sufficient to increase myofilament Ca^{2+}-sensitivity and result in diastolic Ca^{2+}-overload. We speculate that the latter rise of diastolic Ca^{2+} can either result from the high Ca^{2+}-sensitivity that buffers more Ca^{2+} at the myofilaments and/or alterations in the ADP/ATP ratio that reduce the free energy released from ATP hydrolysis (ΔG_{ATP}) of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). Our study supports the idea that elevations of intracellular ADP, in specific types of cardiac disease where myocardial energy reserve is limited, contribute to diastolic dysfunction by recruiting cross-bridges even at low Ca^{2+} ("high Ca^{2+}-state").

High cross-bridge component in HCM

The fact that impaired relaxation is an almost universal finding in heart failure patients, and that this may result from inappropriate formation of force-producing cross-bridges during diastole ("high Ca^{2+}-state") lead us to study the mechanical changes associated with the blockade of actomyosin interactions, i.e. alterations in tropomyosin's position in Chapter 6. To this end, we investigated if the accessibility of myosin-binding sites on actin is altered in human idiopathic dilated cardiomyopathy (IDCM) and HCM samples. By measuring cardiomyocyte force-production in ADP-containing solutions (without Ca^{2+}) without and with exogenous PKA, the ability of myosin-ADP to bind non-blocked sites on actin was assessed. It is important to stress that any alteration in actomyosin interaction at this stage (no Ca^{2+}, but with ADP), precedes changes that occur in the presence of Ca^{2+} as occurs during muscle activation. In other words, an increased contribution of force-producing cross-bridges at the blocked state (B-state) will enhance the number of cross-bridges that are recruited in the presence of Ca^{2+}. Our study supports that diseased muscle has disrupted steric blockade of the thin-filament. This is either caused by the presence of mutant proteins, lack of protein in the case of cMyBP-C haploinsufficiency and/or reduced PKA-phosphorylation of myofilament proteins. Our mechanistic study supports the novel idea that the OFF

to ON transition of the thin-filaments is regulated by PKA-target phosphorylation and cMyBP-C. In addition, we show that, in the absence of Ca^{2+}, troponin mutations increase the actomyosin interactions; in contrast cMyBP-C and PKA both reduce accessibility of myosin-binding sites on actin.

Finally, cardiomyocyte from IDCM and HCM were shown to be more sensitive to Ca^{2+} in the presence of a pathologic level (100 μM) of ADP compared to controls. Exogenous treatment with PKA revealed that, except for troponin T mutations and myosin heavy chain mutations, all HCM samples were normalized to controls. Although PKA reduced myofilament Ca^{2+}-sensitivity in all samples, EC_{50} values in HCM in the presence of ADP (mimicking energy depletion) do not increase to the value observed in non-failing donor myocardium in the absence of ADP (i.e. healthy condition; Figure 1). In other words, our data suggest that even in environments where PKA-phosphorylation is preserved or normalized to controls, Ca^{2+}-sensitivity remains high due to an elevation of ADP. The detrimental effect of ADP in myofilament function is exacerbated when PKA-phosphorylation background is reduced compared to controls. In summary, our data support that enhanced actomyosin interaction contributes to diastolic dysfunction as myofilaments are highly sensitized by the synergistic actions of low phosphorylation of PKA-targets, elevated *in vivo* levels of ADP and of Ca^{2+}.

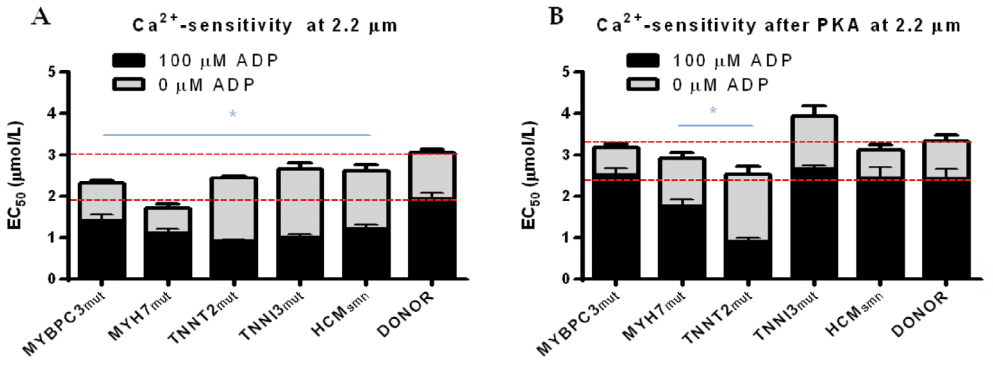

Figure 1. Myofilament Ca^{2+}-sensitivity at a sarcomere length of 2.2 μm. Myofilament Ca^{2+}-sensitivity in the absence (gray bars) and presence of 100 μM ADP (black bars) in hypertrophic cardiomyopathy (HCM) cardiomyocytes. Myofilament Ca^{2+}-sensitivity before (A) and after (B) protein kinase A (PKA). Data was compared using multilevel analysis. *MYBPC3*$_{mut}$, myosin-binding protein-C mutations; *MYH7*$_{mut}$, myosin heavy chain mutations; *TNNT2*$_{mut}$, cardiac troponin T mutation; *TNNI3*$_{mut}$, cardiac troponin I mutation; HCM$_{smn,}$ sarcomere mutation-negative HCM samples. Non-failing hearts (donor) served as controls. Data from Chapter 3 and chapter 6 were combined to assess the effects of calcium in the absence (Chapter 3) and presence (Chapter 6) of a pathologic ADP level. Moreover, these graphs illustrate the effect of hypophosphorylation, which is frequently observed in cardiomyopathy. If we assume that ADP in HCM is high and phosphorylation of myofilament proteins is low, EC_{50} in HCM myocardium would be ~1 μM, compared to ~3 μM in non-failing donor myocardium with high PKA-phosphorylation and no ADP.

CONCLUSIONS

The main message of my studies is that a high level of cross-bridge interaction, which increases myofilament Ca^{2+}-sensitivity, contributes to force development during the diastolic phase. This may impair ventricular relaxation and limit ventricular filling. An optimal therapeutic strategy would be to reduce the 'high Ca^{2+}-state' and in parallel correct low PKA-phosphorylation and the elevated myocardial ADP. As highlighted, the 'high Ca^{2+}-sensitive' state has the potential to slow myocardial relaxation, limit diastolic re-lengthening and heart cavity size. These would reduce the Frank-Starling reserve and may ultimately lead to sudden cardiac arrest. Overall our data suggests that a combination of adrenergic and metabolic treatment might be beneficial for diastolic heart failure patients.

FUTURE PERSPECTIVES

Based on our recent findings published in Chapters 3, 5 and 6, we propose that high myofilament Ca^{2+}-sensitivity ('the high Ca^{2+}-state') is a common characteristic in human HCM, which may associate with high ADP levels. It is currently unclear why HCM patients would suffer from high myocardial ADP, but this may result from depleted myocardial energy reserve. Currently scarce data is available to explain why and how the energetic reserve is reduced in HCM individuals. There are several candidates that deserve focused attention, including creatine kinase (CK) and mitochondrial activity. Unpublished data from our group shows that CK expression is reduced in sarcomere-mutation positive HCM patients (Figure 2A).

This coincides with a substantial reduction in CK-activity compared with non-failing controls (Figure 2B). The change in protein expression was independent of changes that precede CK protein transcription, since mRNA of CK levels were not significantly altered in the majority of HCM samples. The only exception was a reduction in mitochondrial CK (CK-mt) mRNA in thin-filament mutations (Figure 3B).

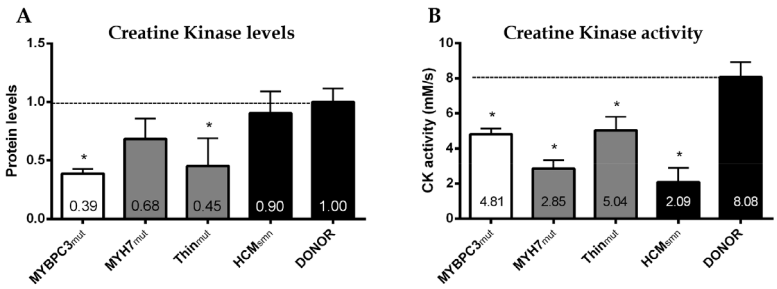

Figure 2. Creatine Kinase (CK) protein-expression (A) and activity (B) in human hypertrophic cardiomyopathy (HCM) samples. Data was compared using multilevel analysis. *MYBPC3*$_{mut}$, myosin-binding protein-C mutations; *MYH7*$_{mut}$, myosin heavy chain mutations; Thin$_{mut}$, Thin-filament mutations consist of cardiac troponin T and cardiac troponin I mutations; HCM$_{smn,}$ sarcomere mutation-negative HCM samples. Non-failing hearts (donor) served as controls. Unpublished data.

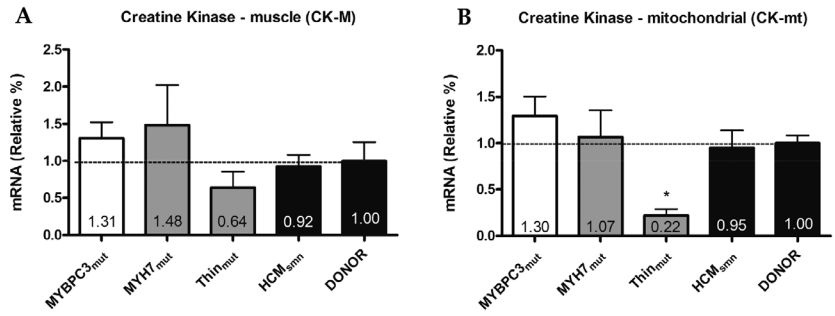

Figure 3. Creatine Kinase (CK) mRNA-expression of muscle (A) and mitochondrial forms (B) in human hypertrophic cardiomyopathy (HCM) samples. Data was compared using 1-way ANOVA analysis. *MYBPC3*$_{mut}$, myosin-binding protein-C mutations; *MYH7*$_{mut}$, myosin heavy chain mutations; Thin$_{mut}$, Thin-filament mutations consist of cardiac troponin T and cardiac troponin I mutations; HCM$_{smn,}$ sarcomere mutation-negative HCM samples. Non-failing hearts (donor) served as controls. Values were set to 1, relative to donor. Unpublished data.

In summary, the present data will strengthen the overall idea about the essential role of myocardial energetic reserve in HCM and will possibly provide alternative areas to carefully study in HCM pathophysiology. Measurements of mitochondrial activity require fresh human HCM samples. We will analyse mitochondrial oxygen consumption in our future studies. In addition, it is imperative to quantitatively measure the precise levels of ADP in the cell, which can already be accomplished nowadays with high-sensitive fluorescent biosensors, such as "PercevalHR". It is our belief that a complex of myofilament and Ca^{2+}-handling changes is triggered by deficient (mitochondrial) energetics in HCM. Future approaches should use animal models and test novel drugs to improve myocardial energetic reserve, including the promising SS-31 (Szeto-Schiller), a mitochondrial-targeted antioxidant peptide that promotes mitochondrial respiration and ADP regeneration to ATP. These have the future potential to partially rescue HCM progression in humans.