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Summary of thesis and
future perspectives



Cardiac remodeling and genotype-specific pathogenic effects in dilated cardiomyopathy

The purpose of this thesis was to elucidate underlying pathological processes in dilated cardiomyopathy (DCM). DCM can have various underlying causes and the patient population is heterogeneous. We used human patient samples obtained during cardiac surgery to study secondary disease remodeling in adult onset DCM, peripartum cardiomyopathy (PPCM) and pediatric cardiomyopathy (pediatric CM). PPCM occurs at the end of pregnancy or within the first months after giving birth¹. PPCM patients show a fast cardiac deterioration and high mortality while complete recovery is also common^{2,3}. Also pediatric CM patients show an early and progressive disease onset but also in this patient population complete recovery is possible⁴. This is contrary to adult onset DCM which often show later age of onset, slower progression, and while stabilization upon treatment is possible, recovery is unlikely^{5,6}. In this thesis we assessed differences and commonalities in secondary disease remodeling in a unique set of human adult DCM, PPCM and pediatric DCM samples. Our approach revealed hallmark pathological changes which may explain their similar clinical phenotype and symptoms, but also unique cellular alterations which may underlie the diverse progression, response to treatment and recovery in DCM, PPCM and pediatric DCM.

In addition to secondary disease remodeling, we assessed genotype-specific pathogenic effects of mutations in the genes encoding cardiac troponin I (*TNNI3*), cardiac troponin T (*TNNI2*), lamin A/C (*LMNA*) and RNA binding motif 20 (*RBM20*). Despite their diverse functions, mutations in various genes result in a similar pathological remodeling leading to the same clinical phenotype of DCM. We assessed the different cellular pathological pathways induced by these mutations and how they contribute to the development of DCM.

Common secondary disease remodeling in dilated cardiomyopathies

During heart failure the heart undergoes cardiac remodeling. Part of this remodeling contributes to disease development in a pathological way. However, another part of this remodeling actually aids in coping with altered cardiac demands and may therefore be beneficial.

β -adrenergic receptor signaling, cardiac troponin I phosphorylation and myofilament Ca^{2+} -sensitivity

In heart failure the β -adrenergic receptor system is overstimulated in an attempt to maintain cardiac performance. While initially compensatory, chronic receptor stimulation becomes detrimental leading to desensitization of the β -adrenergic receptors and subsequent impaired protein kinase A (PKA)-mediated phosphorylation of downstream protein targets which regulate cardiomyocyte contraction and relaxation^{7, 8}. One of these targets is cardiac troponin I (cTnI), and phosphorylation of cTnI modulates myofilament Ca^{2+} -sensitivity. In chapter, 3, 4 and 6 we showed that DCM, PPCM, ischemic heart disease (ISHD) and pediatric CM samples all have hypophosphorylated cTnI and coincident increased Ca^{2+} -sensitivity compared to controls. Since cTnI is phosphorylated by PKA we believe that the observed hypophosphorylation of cTnI is a reflection of secondary disease remodeling in end-stage heart failure. The increase in myofilament Ca^{2+} -sensitivity might compensate for reduced systolic function of the dilated ventricle by increasing the amount of force produced by the myofilaments at a given $[\text{Ca}^{2+}]$. However, this may also lead to unwanted contractions in diastole and impaired relaxation. Moreover, Ca^{2+} -sensitized myofilaments may be a substrate for cardiac arrhythmias⁹.

Titin isoform shift and passive tension

Another feature of cardiac remodeling in DCM is a shift in titin isoform composition. Titin is a giant sarcomeric protein spanning the sarcomere from the Z-disc to the M-line. Titin acts as a molecular spring in which the elastic I band coils up and straightens during contraction. Titin exists in two different isoforms: the stiff N2B isoform and the more compliant N2BA isoform. Both isoforms co-exist in the human heart and a shift in isoform can modulate diastolic function by alterations in passive tension¹⁰. Various studies have shown that compliant titin expression is induced in DCM^{11, 12} and we have confirmed this in chapter 3, 5 and 7 in our patient samples of adult onset DCM and PPCM. However, despite the increase in compliant titin, we observed an increase in passive tension in PPCM patients in chapter 3. Apart from an isoform shift, passive tension can be regulated by PKA-mediated phosphorylation of titin^{13, 14}. We showed in chapter 3 that titin is indeed hypophosphorylated at a PKA-target site and that this was causal to the increased passive tension in PPCM samples. Therefore, although titin can alter passive tension through an isoform shift, post-translational modifications of titin can counteract these effects. In chapter 4 we did observe a decrease in passive tension in pediatric CM samples, however, we also showed that this was not due to titin isoform shift.

Titin, cTnI phosphorylation and blunted length-dependent activation of myofilaments in DCM

In addition to its role in modulation of passive tension, it has been suggested that titin isoform composition plays a role in length-dependent myofilament activation¹⁵. Secondary disease remodeling leading to changes in cTnI phosphorylation and titin isoform composition have a synergistic role in modulating length-dependent activation of myofilaments¹⁶. Length-dependent activation of myofilaments is the ability of the heart to increase contractility upon stretch and provides the cellular basis of the Frank-Starling mechanism. The effect of titin on length-dependent activation was shown in a study comparing bovine atrial tissue to bovine ventricle tissue that showed that the former, which has the most compliant titin, showed a reduced length-dependent activation¹⁵. Another study that showed an increase in compliant titin due to removal of the RNA recognition motif of *RBM20* in mice, showed that compliant titin impairs length-dependent activation¹⁷. However, our studies in human cardiac samples consistently show that the effect of compliant titin on length-dependent activation of myofilaments is limited. Our cardiomyocyte measurements show that the length-dependent increase in myofilament Ca²⁺-sensitivity depends on the phosphorylation status of cTnI in adult onset DCM and PPCM. A blunted increase in length-dependent myofilament Ca²⁺-sensitivity was observed in adult onset DCM and PPCM in chapter 3, which was restored upon incubation with exogenous PKA. This implies that the detrimental effect on length-dependent activation of myofilaments by an increase in compliant titin is limited when cTnI phosphorylation is restored. This was confirmed in pediatric CM in chapter 4 where we observed a slightly larger impairment of length-dependent activation of myofilaments in samples with a higher N2BA/N2B ratio compared to samples with a lower N2BA/N2B ratio although the difference between these two groups was not significant. Both groups showed an impaired length-dependent activation of myofilaments compared to controls. After incubation with exogenous PKA, the difference between the two groups was eliminated and similar to controls. A unique human tissue sample that carried the *RBM20*_{p.E913K} mutation enabled us to deliver a proof-of-concept that compliant titin on its own does not impair length-dependent activation of myofilaments. In chapter 5 we show that this mutation leads to haploinsufficiency of *RBM20* causing an increase in compliant titin isoform composition and size. While length-dependent activation was indeed blunted in cardiomyocytes of this patient, we could also restore it to control levels after incubation with exogenous PKA. An overview of the baseline Ca²⁺-sensitivity and length-dependent activation of myofilaments throughout this thesis and the changes upon incubation with exogenous PKA is shown in Figure 1.

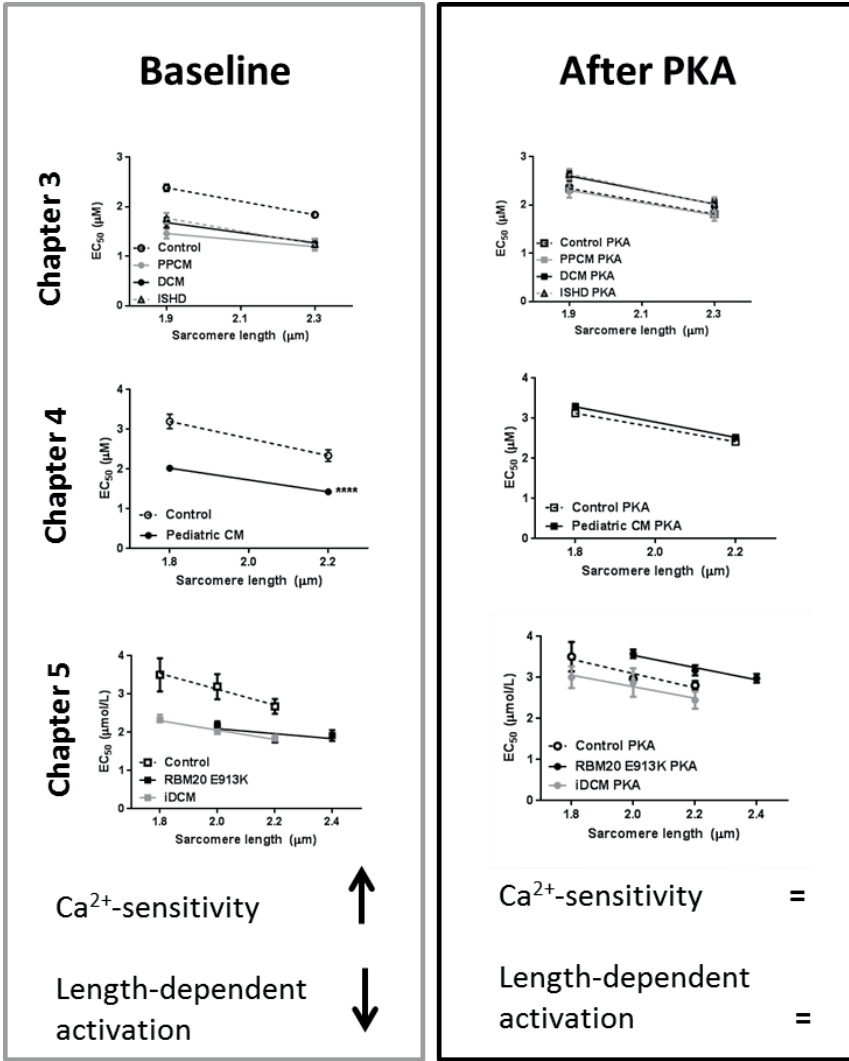


Figure 1. Ca^{2+} -sensitivity, length-dependent activation and changes upon PKA incubation.

Ca^{2+} -sensitivity at baseline is increased in PPCM, DCM, ISHD pediatric CM and DCM patient with RBM20^{p.E913K} mutation. Length-dependent activation, indicated by the slope of the curve, was also impaired at baseline in all groups compared to controls. Upon incubation with exogenous PKA both Ca^{2+} -sensitivity and length-dependent activation was normalized to control levels in all samples.

Titin and exercise

In chapter 4 we found that a higher N2BA/N2B isoform ratio correlated significantly with a smaller reduction in thickness of the LV during systole (LVPWs). During contraction the LV thickens and this thickness in systole is smaller in DCM which is consisted with an impaired systolic function. In pediatric CM we found that all patients indeed showed a reduced LVPWs, but that samples with a high N2BA/N2B ratio came from patients with a smaller reduction in LVPWs, which implies they have smaller reduction in LV thickness during systole compared to patients who had a low N2BA/N2B ratio. This implies that patients with an increased N2BA/N2B ratio have a better LVPWs which is beneficial for systolic function. In addition, a trend was observed between a higher N2BA/N2B and a less dilated ventricle (lower LV end diastolic diameter (LVEDD) and end systolic diameter (LVESD)). Based on these observations, we hypothesize that the increase in compliant titin reflects an adaptive capability of the heart to cope with altered cardiac demands (i.e. counteract high wall stress). This idea is supported by the notion that lower N2BA/N2B ratios were found in patients with a restrictive filling pattern and that impaired diastolic function was associated with a poor prognosis in DCM^{12, 18, 19}. An interesting finding was that *RBM20* mutated mice, in which exon 6 and 7 were deleted leading to deletion of the RNA recognition motif and subsequent large isoforms of compliant titin, showed increased exercise capacity¹⁷. Authors explained this by increase in heart rate during exercise. A higher heart rate can mask the effect of the depressed length-dependent activation of myofilaments during exercise. In addition, due to the higher heart rate during exercise, the sarcomere length is shorter, which might allow titin to still exert its restoring force^{17, 20}. A better exercise tolerance, measured as peak oxygen consumption, positively correlated with N2BA/N2B ratio in DCM patients¹². If indeed the impact of titin isoform composition on systolic function is limited and diastolic function is preserved by an increase in compliant titin, an increase in compliant titin might exert a compensatory effect during cardiac disease progression.

Differential cardiac remodeling in patient populations

PPCM samples show different cardiac remodeling and response to β -adrenergic receptor stimulation compared to adult DCM

Phosphorylation of titin at Ser4010, a PKA-target site, was decreased in PPCM and DCM but not in ISHD (chapter 3) indicating cardiac remodeling is different in DCM and PPCM compared to ISHD. In addition, we observed that PPCM samples have an altered response to PKA-mediated hypophosphorylation than DCM samples. PPCM patients showed increased passive force and a larger increase in Ca^{2+} -sensitivity and blunted length-dependent activation of myofilaments compared to DCM. However,

the reduction in cTnI phosphorylation and phosphorylation of titin at PKA-target site Ser4010 in PPCM was similar to the reduction observed in DCM samples. Therefore, the blunted LDA of myofilaments and the increase in passive tension in PPCM cannot be solely explained by hypophosphorylation of cTnI and modifications in titin. We show in chapter 3 that the different PKA-mediated myofilament response may be due to the lack of re-expression of fetal myomesin in PPCM, a M-line component re-expressed in DCM to provide stability under overstretched conditions. The cardiac remodeling in PPCM patients is therefore slightly different than what is observed in DCM and ISHD and contributes to altered contractility of cardiomyocytes.

Hypocontractility and reduced myofibril density in pediatric CM

While pediatric cardiomyopathy patients showed the same hypophosphorylation of cTnI and increased myofilament Ca^{2+} -sensitivity as observed in adult DCM, pediatric CM samples showed a wide variation in titin isoform composition. While some patients did, others did not have altered titin isoform composition or even showed a shift to more stiff titin (chapter 4).

While we did not observe changes in maximal force development in adult DCM in chapter 3, we did observe a significant reduction in maximal force in pediatric CM samples in chapter 4. Also passive force was significantly reduced in pediatric CM compared to controls. We showed that this was due to a decrease in myofibril density. Combined with the observation that many pediatric patients did not show an upregulation of compliant titin, this could imply that the cardiomyocytes do not undergo extensive remodeling and therefore cannot cope with the cardiac stress. This is in line with two recent publications that showed pediatric CM have limited hypertrophy and interstitial fibrosis compared to adult onset DCM,²¹ and a gene expression profile that supports a more undifferentiated cellular state²². The limited adverse remodeling in pediatric CM might have contributed to their early and progressive disease. Current therapies, mostly focused on treating adverse cardiac remodeling, show highly diverse effectiveness in pediatric CM patients²³⁻²⁵. Only 22% of all patients show full recovery within 2 years²³. It is tempting to speculate that such treatment strategy fails in a large group of pediatric patients who have limited adverse cellular remodeling, and a fast decline in myofibril density which would significantly impair systolic function of the heart. Therapy in these children may rather focus on the regenerative capacity and myofibrillogenesis of cardiac muscle cells rather than reversal of cellular remodeling.

Genotype specific changes in DCM

In 20-50% of patients a mutation is likely to be causal to DCM^{5, 26, 27}. Mutations have been found in various genes ranging from mutations in genes encoding for sarcomeric proteins such as titin (*TTN*), cardiac troponin T (*TNNT2*), cardiac troponin I (*TNNI3*) and tropomyosin (*TMP1*) to genes encoding non-sarcomeric proteins such as lamin A/C (*LMNA*) and desmin (*DES*)⁵. However, it is largely unknown if these genetic variations are actually causal for onset of DCM, and how such diverse mutations can cause the same clinical phenotype. The Exome Aggregation Consortium (ExAC) recently reported that many previously reported rare variants are actually not that rare; they observed that many genetic variants do not occur more often in a DCM population compared with ~60.000 reference samples²⁸. On the other hand, they warned for an underestimation of pathogenicity of variants of uncertain significance (VUS) since some of these were actually significantly more prevalent in the DCM population compared to the ExAC references²⁸. The ExAC study thus underscores the relevance of functional studies to establish pathogenicity of genetic variants. The interpretation of the causality of underlying mutations in DCM is however challenging. Firstly, it is important to distinguish between secondary disease remodeling and mutation-specific pathogenic effects in order to properly counsel family members of DCM patients in which a genetic variation is found. However, distinguish pathogenic effects of mutations from secondary disease remodeling is proven to be a very difficult task in scientific research since not all effects observed in animal models carrying a mutation have to be direct pathogenic effects caused by the mutation. The mutation might cause a subset of pathogenic effects to which the heart responds by general disease remodeling irrespective of the underlying mutation.

***RBM20*_{p.E913K} causes giant titin isoforms and blunted length-dependent activation of myofilaments**

Not all changes in titin isoform composition are due to secondary disease remodeling. For example, mutations in, or knock down of, the splicing regulation of titin; RNA binding motif 20 (*RBM20*), have been shown to result in a DCM phenotype^{17, 29, 30}. Titin is a highly differentially spliced gene. Every titin molecule consists of many Ig domains which can be differently spliced, while the isoform shift from N2B to N2BA is mostly due to the inclusion of a N2A domain and a longer PEVK domain in the N2BA compared to the N2B isoform. However, additional exons can be spliced in or out, thereby affecting the size of titin within an isoform. The main splicing factor involved in this alternative titin splicing is *RBM20*. *RBM20* acts as a splicing repressor: it represses the inclusion of certain exons into the final mRNA molecule³⁰. *RBM20* deficient rats and mice in which the

RNA recognition motif of *RBM20* has been deleted, show inclusion of many additional exons leading to a giant titin isoform called N2BA-G^{17, 29}. In chapter 5 we showed that a patient that carries the *RBM20*_{p.E913K} mutation resulted in haploinsufficiency of *RBM20*, and subsequently increased expression of N2BA. In addition, the N2BA present in this patient was of a size up to 3921 kDa. This is much larger than the N2BA size observed in other DCM patients. For example, in chapter 3 we measured N2BA sizes of 3471, 3446 and 3436 kDa in DCM, PPCM and ISHD, respectively. In addition, the relative increase in N2BA/N2B ratio is of a different magnitude in the patient carrying the *RBM20*_{p.E913K} mutation. In this patient we observed an astonishing N2BA/N2B ratio of 7.45 (chapter 5), while in DCM and PPCM samples (chapter 3) an average ratio of respectively 0.84 and 0.82 was observed compared to 0.52 in controls. Therefore, although many changes in titin can be considered secondary disease remodeling irrespective of underlying mutation, the extent to which these changes occur is severely exacerbated by mutations in *RBM20*. The *RBM20*_{p.E913K} mutation was associated with an increased resting sarcomere length of 1.93 μm compared to 1.81 μm in control cardiomyocytes. This was not due to general secondary disease remodeling, since we show in chapter 3 that resting sarcomere length in DCM is 1.79 μm and in PPCM 1.82 μm . The observed blunted length-dependent activation of myofilaments could be rescued by exogenous PKA, but also unmasked a severely decreased passive tension development in the patient carrying the *RBM20*_{p.E913K} mutation. In addition, functional properties of *RBM20*_{p.E913K} cardiomyocytes were comparable to control cardiomyocytes if the sarcomeres were allowed to function in an 'over-stretched' state³¹. This implies that sarcomeres from this patient might function at a different sarcomere length. However, it remains unknown if the *in vivo* sarcomere length is indeed increased allowing the sarcomeres to operate at this length, or if the sarcomeres are forced to function at a shorter, suboptimal, sarcomere length thereby further impairing contractile properties.

***LMNA*_{p.R331Q} causes unstable lamina leading to decreased myofibril density and hypocontractility**

In chapter 6 and 7 we showed that a missense mutation in the gene encoding lamin A/C, *LMNA*_{p.R331Q} results in decreased myofibril density and subsequent decreased active force development. Since we did not observe a decreased maximal force development in other adult onset DCM samples studied in chapter 3, we believe that this is a mutation-specific pathogenic effect. The region of *LMNA* affected by this mutation is important for homo-dimerization and stability of the nuclear lamina. In addition, the nucleus deforms parallel to the plane of the myofilaments during contraction, and thereby pulls on the nuclear lamina. It is likely that the *LMNA*_{p.R331Q} mutation exerts its pathogenic effect by affecting lamina stability and cytoskeletal integrity leading to decreased

myofibril density and subsequent hypocontractility. The destruction of myofibrils by this mutation is probably a relatively slow process, since these patients have a late disease onset as shown in chapter 6. In chapter 7 we showed that other cardiomyocyte changes observed in the *LMNA*_{p.R331Q} samples such as increased myofilament Ca²⁺-sensitivity could be restored by exogenous PKA and therefore reflect secondary disease remodeling instead of specific mutation-induced effects. This is also in line with the expectation that a mutation in a non-sarcomeric gene is unlikely to affect myofilament function in a direct way.

Mutations *TNNI3*_{p.98trunc} and *TNNT2*_{p.K217del} impair the contractile machinery

Mutations in genes encoding sarcomeric proteins can affect myofilament function in a direct way. In chapter 7 we show that DCM samples with mutations in the genes encoding for cardiac troponin I and cardiac troponin T (*TNNI3*_{p.98trunc} and *TNNT2*_{p.K217del} respectively) had a normal or even increased cTnI phosphorylation compared to what we observed in the *LMNA*_{p.R331Q} samples and other DCM, PPCM or ISHD samples studied in chapters 3 and 7. This might be because the heart is trying to counteract mutation-specific effects. Myofilament Ca²⁺-sensitivity was preserved or even slightly decreased in the *TNNT2*_{p.K217del} sample which is in line with preserved cTnI phosphorylation. However, the *TNNT2*_{p.K217del} mutation exerts its pathogenic effect by an increase in passive force development despite an increase in compliant titin, and preserved PKA-mediated phosphorylation of titin. With troponin exchange experiments we provided proof that high passive force was indeed caused by the *TNNT2*_{p.K217del} mutation. The *TNNI3*_{p.98trunc} sample showed increased Ca²⁺-sensitivity while phosphorylation of cTnI was preserved and exogenous PKA was unable to restore myofilament Ca²⁺-sensitivity (chapter 7). This is clearly different from other DCM samples in chapter 3 and the *LMNA*_{p.R331Q} samples in chapter 7, in which increased Ca²⁺-sensitivity could be restored with exogenous PKA. We showed that the increase in myofilament Ca²⁺-sensitivity in the *TNNI3*_{p.98trunc} sample was due to a decrease in cTnI expression and altered stoichiometry of the troponin complex components. Absence of the truncated cTnI protein in the *TNNI3*_{p.98trunc} sample suggests that the mutated protein is degraded, and the mutation exerts its pathogenic effect through haploinsufficiency. Indeed, exchange experiments to increase cTnI levels to 83% showed this was sufficient to restore Ca²⁺-sensitivity to control levels.

Conclusion

PPCM and pediatric DCM patients show limited and very diverse cardiac remodeling with a highly variable disease progression and outcome ranging from several months between disease onset and need for transplantation or death, to complete recovery. This

is unlike adult DCM which is in general associated with an extensive disease remodeling, slower disease progression, and although the disease in some cases is stabilized upon heart failure treatment, recovery is unlikely. In this thesis we have shown that cardiac remodeling is different in PPCM and pediatric CM compared to adult DCM which might have played a role in the altered disease progression and outcome. What can be learned from the studies in pediatric CM and PPCM is that limited cardiac remodeling and inability to properly build myofibrils might contribute to an aggressive form of cardiac failure. On the other hand, limited cardiac remodeling might also explain why pediatric and PPCM patients can recover completely in contrast to adult DCM in which a slow progressive nature and high levels of cardiac remodeling are associated with an inability to recover. It is tempting to speculate that treatment in pediatric CM should be directed at stimulating myofibrillogenesis rather than reversing cardiomyocyte remodeling.

Apart from secondary disease remodeling genotype-specific effects can contribute to disease development and progression. Secondary changes in titin consist of a shift in isoform composition and post-translation modifications such as phosphorylation, and can be both pathogenic as well as adaptive in DCM pathogenesis. Mutation-specific pathogenic effects of the *RBM20*_{p.E913K} mutation result in a further increase in compliant titin isoform content and size to an extent that is not compensatory but rather disease-causing. A truncation mutation *TNNI3*_{p.98trunc} resulted in haploinsufficiency and altered stoichiometry of the troponin components which led to a reduced length-dependent activation and increased Ca²⁺-sensitivity of myofilaments. While the altered stoichiometry of the troponin components was also observed in a patient with *TNNT2*_{p.K210del} mutation, that mutation also caused increased passive force development. The non-sarcomeric *LMNA*_{p.R331Q} mutation led to decreased myofibril density and subsequent hypocontractility. Therefore, mutations in various genes with various functions can contribute to the same clinical phenotype despite different underlying pathological cellular changes.

Future research

Recent research, including those described in this thesis, show that a disease should not always be considered the same when symptoms are similar. We have shown that cardiac remodeling in pediatric DCM patients, or PPCM patients is different compared to adult onset DCM. A single treatment option for all patient populations is therefore unlikely and have been shown to be ineffective. Since clinical symptoms are often the same in these patient populations, research should focus on the cellular changes underlying these symptoms. Defining which of these cellular processes should be targeted in order to

more effectively treat the disease in a specific population is key. In pediatric patients this could be further investigated by stimulating cardiac remodeling such as an upregulation of compliant titin and hypertrophy in order to prevent myolysis. In PPCM patients future research might focus on the protective EH-myomesin re-expression in order to function under overstretched conditions. Animal models with various expression levels of EH-myomesin in combination with the PPCM STAT3 knock out model might provide answers to this. In addition, also the altered response to β -adrenergic receptor stimulation and PKA-mediated hypophosphorylation in PPCM patients compared to DCM patients warrants further research in order to optimize treatment. The adverse outcome and worsening of heart failure upon treatment with dobutamine in PPCM patients might argue for an entire different treatment approach, or at least a conservative approach in treatment of PPCM patients by manipulating the β -adrenergic system. While treatment with bromocriptine has been shown to be effective in PPCM³²⁻³⁴, this limits the ability of the mother to breastfeed her child. While formula feeding might not be preferred by the mother and breast feeding has been shown to have several health benefits, this might not be a crucial point in infant survival in developed countries. However, taking away the ability to breast feed does provide a severe limitation in survival in underdeveloped countries where PPCM is most prevalent. Alternatively, an anti-oxidant treatment might be developed given the large role of oxidative stress in PPCM³³, or therapies that interfere with the induction of re-expression of fetal genes such as EH-myomesin.

The difficulty to distinguish mutation-specific pathogenic effects from secondary disease remodeling will provide a continuous challenge for future research. In addition, genetic and environmental confounding factors should not be ignored. Also the variable penetrance of mutations is in many cases unexplained so far. A possibility is that a second hit is needed in order to develop the disease. However, what kind of second hit is currently unknown. Possible second hits could be additional mutations, metabolic changes, diet, fitness or in some cases an older age. A new promising technique that could aid in this challenge in the future is engineered heart tissue. These muscle strips of artificially grown cardiac tissue can be made from induced pluripotent stem cells obtained from patient skin tissue. This will allow to study patient specific tissue without the need of a cardiac biopsy. In addition, it also allows the comparison of the effect of a specific genetic variant in patients with different genetic profiles. Combined with the induction of the mutation in induced pluripotent stem cells from healthy controls this can elucidate whether the other genetic factors present in the diseased patients contributed to the observed changes in cardiac function, or that the mutation under investigation was solely responsible. This would not only allow to define with more certainty if the mutation is indeed pathogenic, but also to define contributing genetic

factors that might provide an additional risk to develop the disease. A more thorough understanding of pathogenic variants and additional disease factors would allow for better counseling and treatment options in the future.

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