Cardiomyocyte hypocontractility and reduced myofibril density in pediatric cardiomyopathy

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Submitted
Abstract

Dilated cardiomyopathy amongst children (pediatric cardiomyopathy, pediatric CM) is associated with a high morbidity and mortality. Moreover, treatment based on adult heart failure therapy is often ineffective. However, the reason for high morbidity and mortality is largely unknown as data on cellular pathomechanisms are limited. Here, we assessed cardiomyocyte contractility and protein expression to define cellular pathomechanisms in pediatric CM. For this study explanted heart tissue of 11 pediatric CM and 18 controls was studied. Contractility was measured in single membrane-permeabilized cardiomyocytes and protein expression was assessed with gel electrophoresis and western blot analysis. We observed increased Ca²⁺-sensitivity of myofilaments which was due to hypophosphorylation of cardiac troponin I, a feature commonly observed in adult DCM. Unlike adult DCM we did not find an increase in compliant titin expression. We also found a significantly reduced maximal force generating capacity of cardiomyocytes, caused by reduced myofibril density. The limited ability of pediatric CM patients to induce cardiac remodeling might have contributed to their early disease onset and severity.
Introduction

Dilated cardiomyopathy (DCM) is a cardiac disease characterized by the dilatation of the (left) ventricle and systolic dysfunction. A genetic cause is found in 20-50% of DCM cases(1-3). The disease often develops in adulthood and disease progression can be slow if treatment is optimized though recovery remains unlikely(4). In children, DCM is a rare disease with an annual incidence estimated around 0.6/100 000(5) and it may be associated with an aggressive disease progression. In two large studies the 1- and 5-year transplantation-free survival rates have been reported only 69-74 % and 54-65 % respectively(5, 6). More than 5 years after presentation the rate of events is low(6). Most children die or get transplanted because of pump failure, the rate of sudden cardiac death is relatively low at 2.4% at 5 years(7). Poor prognosis in pediatric DCM patients was associated with thin left ventricular (LV) wall (LV posterior wall thickness z-score < -1.7) and age <13.1 years at time of diagnosis(5-7). In contrast, after 2 years a 22% full recovery has also been reported(8) which implies pediatric cardiomyopathy can also be reversible. The aggressive nature of DCM and the contradictory relative high recovery rate was also reported in a recent Dutch study with a 1- and 5-year survival rate of 85% and 84%, respectively, implying that most patients died within the first year after diagnosis(9). In this study a low transplantation rate in the 1st year after presentation and a 38% recovery rate, of which 50% within 1 year, was reported(9). Determinants underlying the highly diverse response to therapy, recovery rates and mortality are largely unknown. While several pathomechanisms have been elucidated in adult DCM, knowledge on pathogenesis underlying the aggressive progression of DCM at young age is scarce. A recent study showed that pediatric DCM patients harbor a different gene expression profile compared to adult onset DCM(10). pediatric CM samples were characterized by an expression profile that reflected a more undifferentiated cellular state, and reduced hypertrophy response compared to adult DCM(10).

Here we defined the cellular phenotype in a unique collection of pediatric CM samples by combining functional measurements in single isolated cardiomyocytes, protein analyses and electron microscopy. Our studies revealed highly reduced force generating capacity of single cardiomyocytes caused by significant reductions in myofibril density. We observed troponin I hypophosphorylation and an associated increased myofilament Ca\(^{2+}\)-sensitivity and impaired myofilament length-dependent activation. We did not find an increase in compliant titin in the pediatric samples, which is a common form of disease remodeling in various forms of adult heart failure(11-13). However, we did find a large spread in titin isoform composition in the pediatric CM group. Overall, our data indicate that the pediatric CM heart that progresses to end-stage failure has limited capacity to adequately respond to increased wall stress.
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Results

Patient characteristics

Heart tissue was obtained from 9 pediatric patients diagnosed with DCM and 2 pediatric patients diagnosed with non-compaction cardiomyopathy during cardiac transplantation. Z-scores are commonly used to define growth of the heart during development and are used to distinguish between physiological and pathological changes in pediatric patients(14). Patient characteristics are shown in Table 1. LV end systolic diameter (LVESD) and LV end diastolic diameter (LVEDD) were increased and LV posterior wall systole (LVPWs) and LV posterior wall diastole (LVPWd) were decreased, all in line with the dilated phenotype and diagnosis of DCM.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Controls (N=18)</th>
<th>Pediatric CM (N=11)</th>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td>44,1 ± 3,1 years (N=18)</td>
<td>10,5 ± 1,3 years (N=11)</td>
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<tr>
<td><strong>Sex (% male)</strong></td>
<td>55,6% (N=18)</td>
<td>45,5% (N=11)</td>
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<tr>
<td><strong>Time between echo and HTX</strong></td>
<td>15 days (11-64)</td>
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<tr>
<td><strong>LVEDD Z-score</strong></td>
<td>7,9 ± 1.0 (N=11)</td>
<td></td>
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<tr>
<td><strong>LVESD Z-score</strong></td>
<td>11,7 ± 1,0 (N=11)</td>
<td></td>
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<tr>
<td><strong>LVPWd Z-score</strong></td>
<td>-0,8 ± 0,5 (N=11)</td>
<td></td>
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<tr>
<td><strong>LVPWs Z-score</strong></td>
<td>-3,4 ± 0,5 (N=8)</td>
<td></td>
</tr>
<tr>
<td><strong>FS % at presentation</strong></td>
<td>8 (7-10)</td>
<td></td>
</tr>
<tr>
<td><strong>FS % at HTx</strong></td>
<td>15 (8-19)</td>
<td></td>
</tr>
<tr>
<td><strong>NT-pro-BNP at HTx</strong></td>
<td>483 (206-1034)</td>
<td></td>
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</table>

Age, LVEDD, LVESD, LVPWd, LVPWs are shown as mean ±SEM.
Time between echo and HTX, FS% and NT-pro-BNP (pmol/L) are shown as median (interquartile range).
HTX: Cardiac transplantation.

Hypocontractility and increased myofilament Ca$^{2+}$-sensitivity in pediatric CM compared to controls

Measurements in single cardiomyocytes revealed significantly lower $F_{\text{max}}$ (Figure 1A) and $F_{\text{pass}}$ (Figure 1B) in pediatric CM compared to controls. A leftward shift of the force-[Ca$^{2+}$] curve indicated an increased myofilament Ca$^{2+}$-sensitivity in pediatric CM compared to controls (Figure 1C). The combination of reduced $F_{\text{max}}$, reduced $F_{\text{pass}}$ and increased Ca$^{2+}$-sensitivity of myofilaments results in lower force development at high (saturating) [Ca$^{2+}$], lower force at very low [Ca$^{2+}$] and higher force development at intermediate [Ca$^{2+}$]
respectively (Figure 1D). The increase in myofilament Ca^{2+}\text{-sensitivity} was evident at both sarcomere lengths (1.8 μm and 2.2 μm; Figure 1E) and associated with impaired length-dependent activation depicted as ΔEC_{50} (Figure 1F).

**Figure 1.** Baseline characteristics

A: Maximal force was significantly lower in pediatric CM (23.8±1.1, N=11, n=78) compared to controls (37.4±2.6, N=6, n=27, p<0.0001). B: F_{pass} was significantly lower in pediatric CM (N=11, n=40) compared to controls (N=10, n=29, p<0.0001) over a range of sarcomere lengths. C: A leftward shift of the relative force vs [Ca^{2+}] indicates higher myofilament Ca^{2+}\text{-sensitivity} in pediatric CM (N=11, n=38) compared to controls (N=5, n=12, p<0.0001). D: The absolute force development over a range of [Ca^{2+}] showed pediatric CM have impaired maximal force development at saturating [Ca^{2+}], increased force development at lower [Ca^{2+}] and a decreased F_{pass}. E: Ca^{2+}\text{-sensitivity} was significantly higher at sarcomere length 1.8 and 2.2 in pediatric CM (N=11, n=38) compared to controls (N=5, n=12, p<0.0001). F: Length-dependent activation, measured as ΔEC_{50} was significantly lower in pediatric CM (0.60±0.05, N=11, n=38) compared to controls (0.86±0.09, N=5, n=12, p=0.007). N= number of samples, n= number of cardiomyocytes measured.
Figure 2. Titin isoform composition has limited effect on contractility in pediatric CM

A: Separation of titin N2BA and N2B with gel electrophoresis. B: N2BA/N2B ratio was not significantly different between pediatric CM (0.62±0.12, N=11) and controls (0.53±0.03, N=15). C: Length-dependent activation was mostly impaired in pediatric CM patients who had higher N2BA/N2B ratio (N2BA/N2B >0.65, N=5, n=19) compared to pediatric CM patients who had lower N2BA/N2B ratio (N2BA/N2B <0.4, N=6, n=19). Dotted line indicates control values. D: Mean ΔEC_{10} per sample plotted against the N2BA/N2B ratio did not show a significant correlation between ΔEC_{10} and N2BA/N2B. E: There was no difference in F_{pass} between pediatric CM patients who had higher N2BA/N2B ratio (N2BA/N2B >0.65, N=5, n=18) compared to pediatric CM patients who had lower N2BA/N2B ratio (N2BA/N2B <0.4, N=6, n=22). F: N2BA/N2B ratio was not significantly related to age. G: A higher N2BA/N2B ratio was non-significantly associated with a smaller LVEDD z-score. H: A small non-significant trend was observed in which a higher N2BA/N2B ratio was associated with a smaller LVESD z-score. I: No correlation was observed between N2BA/N2B and LVPWd z-score. J: A significant correlation was found in which a higher N2BA/N2B ratio was associated with a less reduced LVPWs z-score (p<0.05). N= number of samples, n= number of cardiomyocytes measured.

Titin isoform composition variation in pediatric CM has limited effect on contractility

A shift towards more N2BA titin has been shown to lower F_{pass} (11, 12, 15) and reduce length-dependent activation (16). Analysis of titin isoform composition did not reveal a difference in the N2BA/N2B ratio between pediatric CM and controls (Figure 2A, B). However, a wide variation in titin isoform composition was observed in the pediatric CM group (Figure 2B). Since titin isoform composition has been shown to affect length-
dependent activation we divided samples into a group that had a high N2BA/N2B ratio (N2BA/N2B>0.65) and a low N2BA/N2B ratio (N2BA/N2B<0.4). The group of samples with a high N2BA/N2B ratio showed a greater reduction in \( \Delta EC_{50} \) than the group of samples with a low N2BA/N2B ratio (Figure 2C). However, \( \Delta EC_{50} \) was not significantly different between the two groups. In addition, \( \Delta EC_{50} \) did not significantly correlate with the N2BA/N2B ratio (Figure 2D). Accordingly, \( F_{\text{pass}} \) was reduced to the same extent in the groups of pediatric CM samples with a relatively high N2BA/N2B ratio (N2BA/N2B>0.65) and a low N2BA/N2B ratio (N2BA/N2B<0.4) (Figure 2E). This indicated that other factors underlie the reduction of \( F_{\text{pass}} \). The wide spread in age of our patient population was not responsible for the spread of titin isoform composition since no correlation was found between age and N2BA/N2B ratio (Figure 2F). We observed a significant correlation between N2BA/N2B and LVPWs (Figure 2J) in which a high N2BA/N2B ratio was associated with a less negative LVPWs z-score. This implies that patients with an increase in compliant titin had a smaller reduction in systolic LV wall thickness.

**Figure 3.** Hypophosphorylation in pediatric CM compared to controls

A: ProQ staining identifying phosphorylated proteins of pediatric CM and control samples. B: Corresponding SYPRO staining identifying proteins of pediatric CM and control samples. C: cTnI phosphorylation is significantly decreased in pediatric CM (N=13) compared to controls (N=13, p<0.0001). D: Phostag showed separation of non-, mono- and bisphosphorylated cTnI. E: While controls (N=11) showed predominantly mono- and bisphosphorylated cTnI, pediatric CM samples (N=11) showed mostly non-phosphorylated cTnI.
Reduced phosphorylation of cTnI in pediatric CM compared to controls

Phosphorylation of cTnI is reduced in various forms of adult heart failure and causes increased myofilament Ca\(^{2+}\)-sensitivity\(^{13, 17-19}\). In line with published data in adult DCM\(^{13, 18, 20}\), phosphorylation of cTnI was significantly lower in pediatric CM compared to controls (Figure 3A,B,C). PhosTag analysis showed separation of non-, mono- and bisphosphorylated cTnI (Figure 3D). While controls showed predominantly bisphosphorylated cTnI, in pediatric CM patients the non-phosphorylated cTnI was more prevalent (Figure 3E). In order to confirm that cTnI hypophosphorylation causes the high myofilament Ca\(^{2+}\)-sensitivity in pediatric CM samples, force measurements were repeated after incubation with exogenous protein kinase A which phosphorylates cTnI. Both myofilament Ca\(^{2+}\)-sensitivity and length-dependent activation normalized to control values upon incubation with exogenous PKA (Figure 4A,B). With normalized phosphorylation status of cTnI, there was no difference in ΔEC\(_{50}\) between the group of pediatric CM samples with a low N2BA/N2B ratio and a high N2BA/N2B ratio (Figure 4C). F\(_{\text{pass}}\) also remained low after incubation with exogenous PKA (Figure 4D). In addition, no difference in F\(_{\text{pass}}\) was observed between PKA-treated groups of pediatric CM samples with different N2BA/N2B ratios (Figure 4E).

**Figure 4. Restoration of sarcomere function after incubation with exogenous PKA**

A: Exogenous PKA restored myofilament Ca\(^{2+}\)-sensitivity in pediatric CM (N=11, n=30) to control (n=5, n=12) values. B: Exogenous PKA eliminated the difference in length-dependent activation (ΔEC\(_{50}\)) between pediatric CM patients with higher N2BA/N2B ratio (N2BA/N2B >0.65, N=5, n=14) than pediatric CM patients who had lower N2BA/N2B ratio (N2BA/N2B <0.4, N=6, n=16) ; relative to control value. Dotted line indicates control values. C: There was no difference between patients with high or low N2BA/N2B ratio with respect to ΔEC\(_{50}\) after incubation with exogenous PKA. D: Exogenous PKA did not affect F\(_{\text{pass}}\) in controls or pediatric CM and remained significantly lower in pediatric CM compared to controls. N= number of samples, n= number of cardiomyocytes measured. E: There was no difference in F\(_{\text{pass}}\) after incubation with exogenous PKA between pediatric CM patients who had higher N2BA/N2B ratio (N2BA/N2B >0.65, N=5, n=13) and pediatric CM patients who had lower N2BA/N2B ratio (N2BA/N2B <0.4, N=6, n=18).
Figure 5. Myofibril density is decreased in pediatric CM

A: Electron microscopy images of 2 control samples and 2 pediatric CM samples. B: Myofibril density was significantly lower (P<0.0001) in pediatric CM (N=10, 45.3%±1.4%) compared to controls (N=10, 57.3%±1.8%). C: F_max normalized for myofibril density of corresponding sample did not differ significantly between pediatric CM (N=10, n=71) and controls (N=6, n=27). D: F_pass normalized for myofibril density of corresponding sample was not significantly different between pediatric CM (N=10, n=37) and controls (N=9, n=25). N= number of samples, n= number of cardiomyocytes measured.
Decreased myofibril density underlies the hypocontractility in pediatric CM

As titin isoform composition was ruled out as causes of the observed decrease in $F_{\text{pass}}$ and $F_{\text{max}}$, we determined myofibril density with transmission electron microscopy. We observed a significant decrease in myofibril density in pediatric CM compared to controls (Figure 5A,B). Correction of $F_{\text{max}}$ for myofibril density eliminated the difference between pediatric CM and controls indicating the hypocontractility was due to myolysis or the inability to create sufficient myofibrils (Figure 5C). Also $F_{\text{pass}}$ of pediatric CM cardiomyocytes was restored to control values after correction for myofibril density (Figure 5D).

Protein quality control system is unaltered in pediatric CM

We then studied whether changes in the protein quality control system occurred, which may underlie reduced myofibril density. Heat shock proteins (HSPs) are upregulated in stressful situations in order to prevent protein denaturation and aid in refolding of misfolded proteins. We did not find an induction of HSP70 (Figure 6A,C) or the cytoskeletal HSP27 (Figure 6B,D) in our pediatric CM group compared to controls. We did find a significantly decreased LC3B1/LC3B-II ratio (Figure 6E,G), which implies an induction of autophagy. However, p62, another autophagy marker was not upregulated in pediatric CM compared to controls (Figure 6F,H).

Discussion

Characterization of pediatric CM myocardium revealed reduced active and passive cardiomyocyte force development, high myofilament Ca$^{2+}$-sensitivity and a blunted length-dependent activation compared to controls. High myofilament Ca$^{2+}$-sensitivity and blunted length-dependent activation were explained by low PKA-mediated phosphorylation of cTnI, which is a general feature observed in cardiac disease. We showed that the decrease in $F_{\text{max}}$ and $F_{\text{pass}}$ is due to decreased myofibril density.

No indications for alterations in protein quality control system in pediatric CM

We observed a decrease in myofibril density which was causal to the hypocontractile cellular phenotype in pediatric CM. Reduced HSP expression has been shown to increase cardiac damage after brief ischemia, and pretreatment with heat to induce HSPs reduced cardiac damage after infarction. However, in conditions of continuous stress as is the case in DCM, the HSP responses are less clear. HSP27 has been shown to be upregulated in adult DCM and not in ischemic heart disease(21). There are conflicting reports about HSP70 in adult DCM ranging from no change(21), to an increased expression(22). HSPs
Figure 6. Protein quality control system in pediatric CM

A: Representative blot images for HSP27 expression. B: Representative blot images for HSP70 expression. C: Expression of HSP27 was not altered in pediatric CM (N=11) compared to controls (N=11). D: Expression of HSP70 was not altered in pediatric CM (N=11) compared to controls (N=11). E: Representative blot images for LC3B-I and LC3B-II expression. G: Expression of LC3B-I/LC3B-II was significantly (p<0.05) reduced in pediatric CM (N=11) compared to controls (N=11). F: Representative blot images for p62 expression. H: Expression of p62 was not altered in pediatric CM (N=11) compared to controls (N=7).
may lose their responsiveness in conditions of continuous cardiac stress. We did not find an induction of heat shock response, and we also did not find more autophagosomes in pediatric CM compared to controls. Future studies are warranted to reveal if reduced myofibril density in pediatric CM is due to an inability of cardiomyocytes to increase myofibril synthesis and/or is caused by increased myofibril degradation.

Low PKA-mediated phosphorylation, high myofilament \( \text{Ca}^{2+} \)-sensitivity and blunted length-dependent activation

In line with what has been found in adult DCM patients\(^\text{(13, 17)}\), we observed decreased PKA-mediated phosphorylation and coincident increased \( \text{Ca}^{2+} \)-sensitivity due to low cTnI phosphorylation in pediatric CM patients. Hypophosphorylation of cTnI has been shown to occur in various forms of heart failure. It is likely due to desensitization of the \( \beta \)-adrenergic receptor signaling pathway and subsequent decrease in PKA-mediated phosphorylation\(^\text{(23)}\). Hypophosphorylation of cTnI has been shown to underlie a blunted length-dependent activation\(^\text{(20, 24, 25)}\). Treatment of cardiomyocytes with exogenous PKA corrected both \( \text{Ca}^{2+} \)-sensitivity of myofilaments and length-dependent activation, independent of the titin isoform composition present in the heart.

Titin isoform composition is highly diverse in pediatric CM

Titin is composed of two isoforms, a compliant N2BA and a stiff N2B isoform. On average we observed no significant change in titin isoform composition in pediatric CM compared to controls. An increase in N2BA/N2B ratio has been reported in various forms of heart failure including adult DCM\(^\text{(11-13)}\) and is considered a general hallmark of DCM. However, in our pediatric CM study population we observed a wide variation of N2BA/N2B ratios: 5 patients showed a higher N2BA/N2B ratio, while 6 patients showed a normal or even lower N2BA/N2B ratio compared to controls. An increase in N2BA titin has been shown to cause a blunted length-dependent activation in animal models,\(^\text{(16, 24, 26, 27)}\) while we only observed a modest effect of N2BA on length-dependent activation of myofilaments in human pediatric CM samples. The length-dependent increase in myofilament \( \text{Ca}^{2+} \)-sensitivity was slightly, though not significantly lower in pediatric CM samples with a high N2BA/N2B compared to samples with a low N2BA/N2B ratio. Length-dependent activation of myofilaments was normalized in all pediatric CM samples to control values after incubation with PKA which indicates that the increase in compliant titin only affects length-dependent activation when cTnI is hypophosphorylated. This is in line with what has been reported in adult DCM\(^\text{(13, 18)}\). While increased N2BA/N2B ratio has been associated with impaired systolic function, a low N2BA/N2B ratio is associated with improved diastolic function and a positive
correlation between N2BA/N2B and peak oxygen consumption, a measure for exercise tolerance, in DCM patients has been found(11). We observed that a high N2BA/N2B ratio coincided with a smaller reduction in LV wall thickness during systole (LVPWs). An increase in N2BA/N2B ratio may represent a compensatory mechanism in order to cope with altered cardiac stress. Overall, the increase in N2BA/N2B did not have a large impact on sarcomere function. However, it should be stated that all studied heart tissue was derived from end-stage pediatric CM patients and therefore might suffer from severe cardiac remodeling. It might be possible that N2BA/N2B aids in this remodeling in a positive way by creating more flexibility in the sarcomeric structure to function under overstretched conditions at the initial disease stage.

**Cardiac remodeling: friend or foe?**

The increase in compliant titin might not have a direct causal role in disease pathogenesis, but may rather represent an adaptive response in order to cope with altered cardiac demand. The inability of pediatric CM patients to upregulate N2BA expression might be a reflection of their limited capability to adapt to altered cardiac demands. This is in line with Patel et al. who recently published limited adverse remodeling in pediatric CM patients compared to adult onset DCM(28). They showed that hypertrophy and perivascular and interstitial fibrosis increased in adult DCM but not in pediatric CM patients compared age-matched controls. In addition, they showed sarcomere thickness is increased in adult DCM, but not in pediatric CM(28). Together with our results this implies that limited cardiac remodeling in pediatric CM patients might hamper the hearts to cope with altered cardiac demands and might have contributed to their early disease onset and severity. However, whether the limited adaptive capabilities of the heart are indeed causal to the early and progressive disease onset warrants further research.

**Limitations**

We have compared pediatric DCM patients with healthy adult controls since acquisition of healthy control tissue of children is near impossible. However, we did not find any correlations between age and protein expression or sarcomere function. Therefore we believe the observed effects are not related to difference in age alone.

Three patients were supported with a ventricular assist device (VAD) prior to transplantation. The duration of VAD support was short, 1-2 months. We did not see a difference in any parameter between patients that were supported with a VAD or not prior to transplantation. However, we can not exclude that despite the short VAD support duration, VAD induced cardiac remodeling.
Conclusion
In summary, we show that pediatric CM patients harbor similar changes in protein modifications and sarcomeric function compared to adult DCM. Hypophosphorylation of cTnI, most likely due to secondary disease remodeling and desensitized β-adrenergic receptor signaling, led to increased Ca\(^{2+}\)-sensitivity and blunted length-dependent activation of myofilaments. However, we did not find a consistent upregulation of compliant N2BA titin as has been observed in adult DCM. Increased N2BA/N2B ratio was significantly related to a lower LVPWs z-score. The limited cardiac remodeling in pediatric CM patients, illustrated in this study by the limited shift in titin isoform composition, might have hampered the ability to cope with altered cardiac demands and might have contributed to their early disease onset and progression. Cardiomyocyte hypocontractility was observed which was due to a decrease in myofibril density. The severe reduction in force generating capacity of cardiomyocytes may underlie the fast progression of cardiac disease in pediatric patients.

Methods
Clinical characteristics
Echocardiographic examinations were performed in a uniform way. All children were at rest and in sinus rhythm during examination and a complete 2-dimensional echocardiographic study was performed. M-mode of the parasternal long-axis was used to measure LVPWs, LVPWd, LVEDD and LVESD and were expressed as Z score for body surface area. Subsequently, FS was calculated using the formula \(((\text{LVEDD}-\text{LVESD}) \div \text{LVEDD}) \times 100\%\).

Cardiomyocyte force measurements
Single cardiomyocytes were mechanically isolated from cardiac tissue and membrane-permeabilized as previously described(29). Maximal force (\(F_{\text{max}}\)) and passive force (\(F_{\text{pass}}\)) of sarcomeres were measured at high \([\text{Ca}^{2+}]\) and low \([\text{Ca}^{2+}]\) (pCa 4.5 and pCa 9.0 respectively). Force-[Ca\(^{2+}\)] curves were constructed at various submaximal \([\text{Ca}^{2+}]\) and are shown as relative forces to \(F_{\text{max}}\). Myofilament Ca\(^{2+}\)-sensitivity was measured as the \([\text{Ca}^{2+}]\) needed to achieve 50% of \(F_{\text{max}}\) (EC\(_{50}\)). length-dependent activation was measured as the shift in EC\(_{50}\) (ΔEC\(_{50}\)) at a sarcomere length of 1.8 μm and 2.2 μm.

Titin expression and cTnI phosphorylation
Titin isoforms were separated on a 1% w/v agarose gel and stained with SYPRO Ruby protein gel stain (Invitrogen) as previously described(30). All samples were measured in
triplicate and average of triplicate measurement per sample is shown. Phosphorylation of cTnI was assessed as previously described(31, 32).

**HSP27, HSP70, LC3B-I/II and p62 expression**

For HSP70 and HSP27 analysis proteins were separated on pre-cast 10% criterion gels (BioRad) and membranes were incubated with HSP70 antibody (Enzo) or HSP27 antibody (Cell Signaling), and GAPDH antibody (Cell signaling) to correct for loading differences. For LC3B-I and LC3B-II analysis proteins were separated on pre-cast 8-16% gradient TGX gels (BioRad) and membranes were incubated with LC3B-I/II antibody. Analysis of p62 expression was performed by separating proteins on a 12% acrylamide gel and membrane was cut in two pieces. The upper piece was incubated with p62 antibody (Cell signaling) and the lower piece with GAPDH antibody.

**Electron microscopy**

Cardiac tissue of pediatric CM and control samples were studied with transmission electron microscopy. Myocardium was fixed in 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), embedded in Epon and cut in 70 nm sections. The sections were mounted onto formvar-coated copper grids and stained with a 5% solution of uranyl acetate, followed by Reynold's lead citrate. Sections were viewed with Philips CM100 Transmission Electron Microscope. The myofibril density was determined with ImageJ software and expressed as percentage of myofibrils of whole cell area in that picture of which the nucleus was excluded. For each sample 2-7 different images were analysed in order to determine average myofibril density. Maximal, and passive forces were corrected for average myofibril density of the corresponding sample.

**Statistics**

Graphpad Prism v7 software was used for statistical analysis. Means were compared between groups with T-test if data was confirmed to be normally distributed. Maximal force data was not normally distributed and therefore means were compared with a Mann-Whitney test. A p-value<0.05 was considered to represent a statistically significantly difference between groups. N= number of samples, n= number of cardiomyocytes measured.
Chapter 4

Ethical approval
This study was approved by the local ethics board of the Erasmus Medical Center (protocol number MEC-2015-233) and written consent of patients and/or parents was obtained. Samples were obtained during cardiac transplantation. As we do not have access to control cardiac tissue from age-matched individuals, 18 control samples were used from explanted Left ventricular (LV) heart tissue of healthy donors (age range 21 to 65 years old); people died from a non-cardiac cause, typically motor vehicle accidents and were acquired from the University of Sydney, Australia, with the ethical approval of the Human Research Ethics Committee #2012/2814. The codes of used samples are: 6.034, 8.004, 5.086, 3.141, 3.164, 4.049, 4.104, 7.040, 6.020, 5.128, 3.160, 6.008, 7.054, 7.044, 6.056, 3.112 6.042 and 3.162. All samples were stored in liquid nitrogen or at -80°C until use.

Author contributions
IB performed and analyzed the contractile force experiments and titin isoform composition analysis. IB imaged samples with TEM and analyzed myofibril density. IB performed overall data interpretation and manuscript production. MM and MD acquired patient samples and clinical data and assisted with data interpretation and manuscript production. KG performed and analyzed protein phosphorylation experiments. DK and JV were involved in overall study design, data interpretation and manuscript production.

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References


