Structural and Functional Cardiac Profile after Prolonged Duration of Mechanical Unloading: Potential Implications for Myocardial Recovery

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Abstract

Clinical and experimental studies have suggested that the duration of left ventricular assist device (LVAD) support may affect remodeling of the failing heart. We aimed to (1) characterize the changes in calcium/calmodulin-dependent protein kinase type II (CaMKII)δ, growth signaling, structural proteins, fibrosis, apoptosis and gene expression before and after LVAD support and (2) assess whether the duration of support correlated with improvement or worsening of reverse remodeling. LV apex tissue and serum pairs were collected in patients with dilated cardiomyopathy (n=25, 23 male) at LVAD implantation and after LVAD support at cardiac transplantation/LVAD explantation. Normal cardiac tissue was obtained from healthy hearts (n=4) and normal serum from aged-matched controls (n=4). The duration of LVAD support ranged from 48 to 1170 days (median duration 270 days). LVAD support was associated with CaMKIIδ activation, increased nuclear myocyte enhancer factor-2 (MEF2), sustained histone deacetylase-4 (HDAC4) phosphorylation, increased circulating and cardiac myostatin (MSTN) and MSTN signaling mediated by SMAD2, ongoing structural protein dysregulation and sustained fibrosis and apoptosis (all p<0.05). Increased CamKIIδ phosphorylation, nuclear MEF2, cardiac MSTN significantly correlated with duration of support. Phosphorylation of SMAD2 and apoptosis decreased with shorter duration of LVAD support but increased with longer duration of LVAD support. Further study is needed to define the optimal duration of LVAD support in patients with dilated cardiomyopathy.

NEW & NOTEWORTHY

A long duration of left ventricle assist device (LVAD) support may be detrimental for myocardial recovery, based on myocardial tissue studies in patients with prolonged support showing significantly worsened activation of CamKIIδ, increased nuclear MEF2, increased MSTN and its signaling by SMAD2 and apoptosis, as well as sustained HDAC4 phosphorylation, structural protein dysregulation and fibrosis.
Keywords

Mechanical support; left ventricular assist device (LVAD); reverse remodeling; heart failure
**INTRODUCTION**

Mechanical support through left ventricular assist devices (LVAD) is increasingly used as a bridge to heart transplantation and as a destination therapy as an alternative to transplantation. Newer devices like the HeartMate II (Thoratec, Corp., Pleasanton CA) and HeartMate 3 provide increased survival time and quality of life over first-generation devices (27). They also offer extended durability, allowing patients to be supported for longer periods of time.

Heart failure (HF) is characterized by overload-induced structural, molecular and function alterations. These changes, known as cardiac remodeling, promote HF symptomatic progression, ultimately leading to LV enlargement, reduction in contractility and increased intracardiac pressure (7). LVAD support unloads the failing heart and helps restore left ventricular ejection fraction (LVEF), improving cardiac output and organ perfusion. LVAD support may cause reverse remodeling by inducing regression of cardiomyocyte hypertrophy, stopping apoptotic cell loss, recovering normal calcium cycling and lipid metabolism and reducing inflammatory markers and fibrosis (4,10,18). Even in the absence of myocardial recovery that allows weaning from the LVAD, partial recovery of several cardiac remodeling markers due to LVAD support is usually evident (4).

Clinical and experimental studies have suggested that the duration of mechanical unloading may affect the remodeling of the failing heart (23,24,28,29). Therefore, the duration of LVAD unloading must be taken into consideration when interpreting associations between biologic parameters and clinical outcomes. This may be of particularly importance when determining the appropriate time to wean patients with potential for myocardial recovery. We hypothesize that a longer duration of support would negatively influence reverse remodeling and cardiac function in patients supported with LVAD therapy.
There are numerous pathways that may be involved in this process (Figure 1).

Calcium/calmodulin-dependent protein kinase type II (CaMKII) and specifically its predominant cardiac isoform, δ, is a central mediator of cardiac stress (2). CaMKII senses abnormal intracellular Ca\(^{2+}\) and activates by binding calcified calmodulin (CaM). Active CaMKII\(\delta\) activates class IIa histone deacetylases (HDACs), HDAC4 and 5, which in turn activate myocyte enhancer factor-2 (MEF2) transcription factor (3). MEF2 drives the expression of fetal cardiac and stress response genes, including the negative growth factor regulator myostatin (MSTN) (33). MSTN exerts anti-hypertrophic/pro-atrophic signaling, and may be involved in wall thinning in end-stage HF. SMAD2/3 is the main signaling effector of MSTN signaling. Mitogen-activated protein kinase (42/44 MAPK or ERK1/2) is one of the main signaling pathways involved in cardiac hypertrophy and HF, and it is conversely involved in MSTN MEF2-dependent activation (6). SMAD2,3, ERK1/2 as well as AKT and MAP kinase p38 signaling branches regulate extracellular matrix remodeling, cell growth and hypertrophy gene expression downstream from MSTN and in cardiac stress.

Thus, the aims of this study were 1) to characterize the changes in CaMKII\(\delta\) signaling, growth signaling, structural proteins, fibrosis, apoptosis and gene expression in LVAD patients, comparing their pre-implantation levels with those after support and healthy patients, and 2) to assess whether the duration of support correlated with improvement/worsening of reverse remodeling markers and clinical state. This information is critical in helping to understand the optimal duration of support needed for maximal biologic recovery.

MATERIALS AND METHODS

Study design

This study met all institutional guidelines of the Institutional Review Board of Columbia University and New York State organ donation guidelines regarding informed consent, the use
of clinical data, ethical treatment of patients adhering to the Declaration of Helsinki principles, and procurement of tissue for research. All subjects were recruited at the New York Presbyterian Hospital-Columbia University campus between 2010-2012.

**Normal patients**

Subjects with no known cardiopulmonary disease whose organs were listed but were unable to be placed at the time of organ recovery for heart transplantation and who consented to tissue for research purposes by the New York Organ Donor Network were included in this study (n=4) as previously described (8). Serum was obtained from age-matched, healthy persons with no known heart disease (n=4).

**LVAD patients**

Patients with end-stage dilated cardiomyopathy (DCM) who met institutional criteria for LVAD device implantation at the New York Presbyterian-Columbia Campus were included in this study and cardiac tissue samples (n=15) were processed as previously described (10). Paired serum was obtained from a different set of patients at the time of LVAD implantation/explantation (n=10). LVAD type consisted of pulsatile (n=4), axial flow (n=14), and centrifugal pump (n=7). LV end-diastolic dimension (LVEDD) and interventricular septum (IVS) were measured by echocardiography. LV ejection fraction (LVEF) was calculated by the simplified Quinones equation and LV mass was calculated using the Devereux formula. For the study of the influence of LVAD duration as a categorical variable, patients were stratified into short or long duration of support as determined by the median duration of LVAD support in the cohort (270 days, cardiac tissue samples: short support n=7, long support n=8; serum samples: short support n=5, long support n=5).

**Protein analysis**
Lysates of heart tissue (total, nuclear and cytoplasmic fractions) were prepared and analyzed by western blot as previously described (10). The following proteins were analyzed using the indicated antibodies: CaMKII (pan, Cell Signaling, Danvers, MA, #3362), Thr 286 phosphorylated (P-) CaMKII (Cell Signaling, #3361), Thr 305 P-CaMKII (One World Lab, San Diego, CA, A0005), CaM (Abcam, Cambridge, MA, ab45689), MEF2 (Abcam, ab64644), HDAC4 (Abcam, ab1437), HDAC5 (Abcam, ab1439), P-HDAC4 (Abcam, ab39408), P-HDAC5 (Santa Cruz, Santa Cruz, CA, sc101692), MSTN propeptide (R&D Systems, Minneapolis, MN, MAB7881), activin receptor type IIBR (ACTIIBR, Abcam, ab128544), bone morphogenic protein-1 (BMP1, R&D Systems, MAB1927), SMAD2/3 (Cell signaling, #3122), P-SMAD2 (Millipore, Billerica, MA, AB3849), activin A (Abcam, ab89307), P-p44/42 MAPK (phosphor-ERK1/2) (Cell Signaling, #9101), p44/42MAPK(ERK1/2) (Cell Signaling, #9102), p38MAPKinase (Cell Signaling, #9212), P-p38MAPKinase (Cell Signaling, #9211), P-AKT (Cell Signaling, #9271), AKT (Cell Signaling, #9272), mechanistic target of rapamycin (mTOR, Cell Signaling, #2972), P-mTOR (Cell Signaling, #2971), corticotropin releasing factor receptor-1 (CRFR1, Abcam, ab59023), CRFR2 (Abcam, ab104368), desmin (Sigma, #D1033), smooth muscle myosin heavy chain (MHC SM, Abcam, ab124679), and matrix metalloproteinase-9 (MMP9, Abcam, ab38898). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Santa Cruz Biotechnology Inc., sc-32233) was used as a loading control for total and cytoplasmic fractions and Lamin A/C for nuclear fractions (Santa Cruz, sc-7292).

**ELISA**

Serum concentrations of myostatin (MSTN) were determined by ELISA using a commercial kit from R&D Systems (Minneapolis, MN, #DGDF80), following manufacturer’s directions. The antibody presents a <0.5% cross-reactivity with growth differentiation factor-11 (GDF11) and other related molecules.
Fibrosis and nuclear DNA fragmentation

Paired pre- and post-LVAD cardiac tissue samples were prepared and analyzed for fibrosis and DNA fragmentation as previously described (10).

RNA library preparation, sequencing, and data processing

Bar-coded RNA libraries were prepared from 3μg total RNA of each LV sample with TrueSeq RNA Sample Prep Kits (Illumina) in accordance with the manufacturer’s recommendations. Bar-coded libraries were mixed and pooled into equimolar (10 nmol/L) amounts and diluted to 4 pmol/L for cluster formation on a single-flow cell lane, followed by pair-end sequencing on an Illumina HiSeq 2000 sequencer.

RNA sequencing analysis

The paired end 100bp sequencing reads were aligned to the GRCh38 reference assembly using the software STAR (14) version 2.5.3a. Aligned reads were mapped to ensemble genes using the R software (https://www.r-project.org/)(version 3.3.1), specifically the package Rsubread version 1.16.1. Differential expression was calculated with the R software (version 3.3.1), specifically the package DESeq2 version 1.16.1.

Data Analysis

The adequacy of our sample size was tested prior by power analysis. Based on a preliminary experiment, we defined cardiac MSTN protein level mean and standard deviation in healthy and HF hearts. Assuming a power of 0.8 with and a significant level of 0.05, a sample size of 4 healthy hearts and 15 DCM patient samples provides a 80-90% chance to detect a 20% difference in cardiac MSTN protein levels using t-testing. Continuous variables are presented as means and standard errors, and categorical data are presented as counts.

Molecular variables were compared using one-way ANOVA with post-hoc Bonferroni analysis.
We explored the effect LVAD support duration, both as a continuous variable and as a
categorical variable (<median duration, >median duration) on clinical and molecular
measurements. Using the duration of LVAD as a categorical variable, we performed mixed-
design ANOVA with “LVAD” as a within-subjects factor and “LVAD duration” as a between-
subjects factor followed by Bonferroni post-hoc test to compare the effect of LVAD and duration
of support on clinical measurements before and after LVAD. Simple linear regression analysis
was used to determine the effect of LVAD time as a continuous variable in clinical and
molecular measurements. Pearson’s product-moment correlation coefficient was used to
determine the correlation between clinical and molecular measurements. We performed
separate ordinary least-squares multivariate linear regression analyses, using the change in
clinical (LVEF, LV mass, LV end-diastolic dimension (LVEDD), brain natriuretic peptide (BNP))
and molecular measurements with LVAD as the dependent variables, to assess the association
between LVAD duration and the measurement change adjusting for age, device type,
ischemic/non-ischemic etiology, HF duration, hypertension, diabetes, previous myocardial
infarction and coronary artery disease. T-testing was used to compare effect on LVAD duration
as a categorical variable in the change in molecular measurement with LVAD. For all analyses,
*p-values were two-sided and a *p*<0.05 was considered significant. All data were analyzed
utilizing SPSS 22 (SPSS, Chicago, IL).

**RESULTS**

**Demographic and clinical data**

Table 1 shows patient demographic and clinical data for the complete cohort and
subdivided by LVAD support duration (<median duration, >median duration). All patients were
maintained on a strict HF medication regimen. The duration of LVAD support ranged from 48 to
1170 days. The LVEF of the patients was significantly increased after LVAD, indicating partial
recovery of contractile function. A decrease of the LVEDD after LVAD support demonstrated effective unloading of the LV. LV mass by echo was significantly decreased after LVAD support. Serum BNP was drastically decreased after LVAD support, consistent with evidence of reverse remodeling. Duration of LVAD did not have an independent effect by mixed-design ANOVA and there was no interaction between LVAD support and duration for any of the clinical parameters studied. Bonferroni post-test indicated a significant change in LVEF in the short support group (difference = 12.5, t = 2.35, p<0.05) while the effect in the long support group was not significant (difference = 7.2, t = 1.51, p>0.05). LV mass change was similarly only significant in the short support group (short support: difference = -168, t = 3.3, p<0.01; long support: difference = -94, t = 1.89 p>0.05). BNP change was significant in both short and long support groups, but this effect was greater in the short support group (short support: difference = -758, t = 4.8, p<0.001; long support: difference = -451, t = 3.5 p<0.01). None of the clinical parameters analyzed showed a significant correlation with duration of support, although there was a tendency for less LV mass reversal with longer duration of support ($R^2=0.14$, p=0.06). No patient demonstrated heart myocardial recovery sufficient for LVAD explantation without transplantation.

**CamKIIδ**

Figure 2 shows changes in CaMKII activation due to LVAD support and the effect of LVAD duration in these changes. After CaMKII has been activated by CaM binding, phosphorylation at Thr287 on CaMKII results in autonomous activity that retains enzymatic activity independent of CaM binding. Increased activity of total CaMKIIδ, as reflected by increased phosphorylation of Thr287, was seen in HF and maintained after LVAD support (Figure 2A). When we stratified patients into two groups (<270 days, >270 days) the change in the activation of CaMKIIδ tended to be dependent on length of LVAD support (Figure 2B): in patients with short duration of support, the activation of CamKIIδ remained unchanged, whereas in patients with long duration of support, activation of CaMKIIδ increased (p=0.05). Moreover,
when we considered duration of support as a continuous variable the change in CaMKIIδ correlated with the duration of support ($R^2=0.27$, $p=0.043$). Subcellular variants of CaMKIIδ have different functions: cytoplasmic CaMKII promotes DCM while nuclear CaMKIIδ promotes cardiac hypertrophy (40). Besides Thr287, CaMKII can be also phosphorylated at Thr305/306: phosphorylation at Thr305 blocks CaM rebinding acting as an auto-inhibitory mechanism. Our results suggest increased overall cytoplasmic CaMKIIδ activity in HF and post LVAD, reflected by increased phosphorylation at Thr287 (Figure 2C) and at Thr305 (Figure 2D), and increased CaM (Figure 2E). Nuclear CamKII is less sensitive to Ca$^{2+}$ levels than cytoplasmic CaMKII; in the nucleus (Figure 2F,G) we found a lack of activation of nuclear CaMKIIδ despite increased nuclear CaM levels (Figure 2H).

CamKIIδ-dependent transcriptional signaling

CaMKII phosphorylates HDAC4 and HDAC5 as a complex, leading to activation of MEF2 transcription factor (3). In their un-phosphorylated state, HDAC4 and HDAC5 inhibit MEF2 activity. Phosphorylation of HDAC 4/5 by CaMKII results in translocation of HDAC4/5 outside the nucleus, hence activating MEF2-mediated transcription (see Figure 1). Phosphorylated levels of HDAC4 were increased in HF and post LVAD, while changes in HDAC5 phosphorylation were not significant in DCM or after LVAD (Figure 3A-D). Cytoplasmic MEF2 was decreased in HF and further after LVAD (Figure 3E), while nuclear MEF2 was increased in HF and further post-LVAD (Figure 3F), suggesting its translocation into the nucleus. There was a trend for nuclear MEF2 change to be stronger in the group patients with longer duration of support ($p=0.06$) (Figure 3G) and the increase in MEF2 after LVAD significantly correlated with the duration of support ($R^2=0.37$, $p=0.015$).

Myostatin
Circulating serum MSTN propeptide (marker of MSTN activation) levels were significantly higher in DCM patients (Figure 4A) and continued to rise after LVAD support compared to normal controls. The change in circulating MSTN levels significantly correlated with the duration of support ($R^2=0.50$, $p<0.0001$). Cardiac MSTN propeptide levels (Figure 4B) were significantly higher in failing hearts than normal hearts and significantly increased after LVAD support. A longer duration of support was associated with an increased in cardiac MSTN (Figure 4C) and these changes significantly correlated with the duration of support ($R^2=0.23$, $p=0.015$). The TGFβ superfamily member activin A shares some redundant actions with MSTN and was similarly higher in patients with HF and LVAD versus control (Figure 4D). MSTN/activin A receptor Activin IIBR was similarly elevated in pre and post LVAD samples (Figure 4E). Cardiac BMP1, responsible for MSTN activation, was increased in HF patients and further increased after LVAD (Figure 4F). A longer duration of support was associated with a tendency ($p=0.06$) towards increased BMP1 levels (Figure 4G) and BMP1 significantly correlated with duration of support ($R^2=0.61$, $p=0.0009$). Interestingly, BMP1 change correlated with changes in LV mass: patients with a higher increase in BMP1 levels after LVAD had lesser degree of LV mass reversal ($R^2=0.33$, $p=0.03$, Figure 4H). SMAD2/3 is the main signaling effector of MSTN signaling and its phosphorylation indicates MSTN signaling. P-SMAD2/SMAD2 levels were elevated compared to control patients in DCM subjects and after LVAD (Figure 4I). While P-SMAD2/SMAD levels tended to decrease in the short support group a longer duration of LVAD support was associated with higher P-SMAD2/SMAD signaling (Figure 4J). The multiple linear regression analysis revealed that another confounding variable explaining P-SMAD2/SMAD2 change with LVAD besides the duration of support was device type: support by axial flow LVAD had a significant effect on P-SMAD2/SMAD2 change (coefficient = 2.36, $p=0.04$). Increased SMAD2 activation, BMP1 levels and increased MSTN propeptide strongly suggests the net effect of longer support is increased MSTN-related signaling.
Stress and growth-related proteins

Cardiac P-ERK42/ERK42 and P-ERK44/ERK44 ratios were increased in DCM patients and significantly decreased with LVAD support (Figure 5A,B). Phosphorylation of the stress MAP kinase p38 levels remained unchanged in DCM and after LVAD support (Figure 5C). AKT phosphorylation, which indicates pro-hypertrophic signaling, tended to be decreased after LVAD support (Figure 5D p=0.07). The phosphorylation of the cell growth regulator mTOR was increased in DCM and after LVAD (Figure 5E), supporting increased protein turnover in DCM with LVAD support. Stress marker CRF1 was increased in DCM and significantly further increased in LVAD support (Figure 5F). CRF2, involved in AKT and ERK activation (36), followed the opposite pattern (Figure 5G). Altogether, these results suggest ongoing hypertrophic signaling in DCM patients with a tendency to decrease after LVAD support. None of the stress and growth-related proteins studied was affected by the duration of support.

Structural Proteins, fibrosis and apoptosis

Actin (Figure 6A) and SM MHC (Figure 6B) levels were significantly higher in HF and sustained after LVAD support. Desmin, which is a marker of hypertrophy, was decreased after LVAD support (Figure 6C), suggesting a lack of pro-hypertrophic activation after LVAD. MMP9, a proteinase central to extracellular matrix remodeling, was significantly increased in HF and after LVAD support (Figure 6D). Changes in the structural markers studied were not dependent on the duration of LVAD support. Fibrosis was present in failing hearts but was unchanged with LVAD support (Figure 6E,F). TUNEL staining showed ongoing apoptosis in HF (Figure 6G,H), a mild decrease in patients supported by shorter periods of time (Figure 6I), correlating with the duration of LVAD support ($R^2=0.23$, p=0.012). These results suggest that a shorter duration of support may be optimal to prevent cell loss.

RNA sequencing
We identified 72 protein-coding RNA sequences (full list in Supplementary Results), which consisted in 59 sequences expressed at significantly higher and 13 sequences expressed at significantly lower levels after LVAD support than before LVAD support. Figure 7 shows the most over- and under-expressed protein-coding sequences after LVAD support vs. before LVAD support. Eight of the genes significantly modified after LVAD support were related to MSTN/SMAD signaling (GADD45α, BMP2, DACT2, PLAU, BMF, RANBP3L, ADAMTS15 and TAL1). The calcium-sensing gene HPCAL4 was significantly increased after LVAD support. C-type natriuretic peptide (NPPC) gene was significantly decreased after LVAD. Changes in RNA abundance did not correlate with the duration of support.

**DISCUSSION**

In the current study LVAD support was associated with sustained activation of CaMKIIδ, HDAC4, nuclear MEF2, MSTN and its signaling mediator SMAD2, mTOR, markers of structural remodeling, fibrosis and apoptosis. Moreover, we provide preliminary evidence that a longer duration of LVAD support correlates with the exacerbation of cardiac stress signaling markers: CaMKIIδ, MEF2 activation, circulating and cardiac MSTN levels, MSTN signaling as reflected by SMAD2 activation, BMP1 cardiac levels and apoptosis increased with longer duration of LVAD support. A shorter duration of LVAD was associated with a decrease in SMAD2 activation and apoptosis.

In our cohort of patients, decreased LV mass and BNP levels after LVAD support indicated reverse remodeling, but these parameters did not translate into enough functional improvement to prevent cardiac transplantation. Despite the well-documented reverse remodeling which is often associated with LVAD a great number of HF patients are refractory to any functional improvement, likely due to the end-stage nature of the disease stage at LVAD implantation (24). In this study, LVAD support was not accompanied by reversal of remodeling.
markers: only P-ERK was partially reversed by LVAD support. RNA seq analysis comparison between pre and post LVAD paired samples evidenced profound gene expression changes due to mechanical unloading, however, the clinical significance of these changes is difficult to interpret. It has been previously reported that only 5% of dysregulated genes in HF revert back to normal following LVAD support, and in animal models of myocardial recovery only a small subset of HF genes revert back to normal expression patterns despite normalization of the HF phenotype (34). It’s been postulated that persistence of HF transcriptome in the phenotypically recovered myocardium may make these hearts more susceptible to hypertrophic growth (34).

In this study, when patients were stratified according to duration of support a tendency towards recovery in patients supported for shorter periods was revealed: recovery of LVEF, LV mass, and BNP levels was greater in patients supported for shorter periods of time. A growing volume of literature is focusing on the potential effect of that LVAD duration on the clinical outcome of the support. Data from the LVAD Working Group study revealed that LVEF improves initially upon LVAD implantation but falls significantly after 120 days of support, suggesting that prolonged support may not be the best strategy for function recovery (26). Previous studies have suggested a detrimental effect of prolonged LVAD support in recovery of molecular markers. Madigan et al. reported that maximum structural reverse remodeling is complete by 40 days (23). Improvement of Ca\(^{2+}\) homeostasis was achieved with short duration of LVAD while longer support duration offset any early gain afforded by short-term unloading (27). These timelines may not only have important implications for cardiac recovery, they are also expected to worsen arterial function and, thus, contribute to a vicious circle. It is known that pulsatility is a key factor in arterial health (17,37). If LVEF falls after 120 days of support (23), pulsatility is expected to fall concomitantly (9). In LVAD patients, this will further exacerbate the aortic stiffness reported previously (1,30) and, although reversible with heart transplant, likely
increase the risk of adverse complications (21). Although speculative, such arterial alterations may not only worsen overall outcome, they may also further attenuate myocardial recovery and may possibly explain the lack of progression beyond 120 days.

The mechanism by which LVAD support induces reverse remodeling is still not fully understood. It has been postulated that it may involve the same cellular mechanism regulating pressure/volume overload-induced hypertrophy but working in the opposite direction (23). In this sense, reversal of all these signaling pathways would lead to cardiac atrophy. Based on our findings, we postulate that the greatest functional recovery could be achieved by downregulation of pro-atrophic signaling, which may be involved in wall-thinning (MSTN signaling), together with sustained activation of hypertrophic pathways, which would lead to compensatory hypertrophy and improved function. Our results indicate that unload may initially attenuate atrophic processes (P-SMAD2) and apoptosis, thus leading to function recovery as reflected by greater improvement of EF and LV mass. Longer LVAD support may result in an increase of pro-atrophic (MSTN, BMP1) and stress markers (CaMKIIδ, MEF2). In contrast, hypertrophic pathway marker levels (ERK, AKT, p38, mTOR, CRF1, CRF2) and structural markers (actin, SM MHC, desmin) did not appear to be sensitive to LVAD support duration in our study. Similarly, fibrosis and ECM remodeling marker MMP9 continued elevated after LVAD support both in short and long LVAD support groups. The absence of workload during LVAD support may result in disuse atrophy, preventing compensatory hypertrophy activation (AKT, ERK, p38) needed for function recovery. The lack of AKT and p38 activation, the decrease in P-ERK and the unchanged mTOR levels after LVAD found in our study had been previously reported (31,38). The decrease in CRF2 found after LVAD may contribute to the lack of activation of ERK and AKT (36). An attempt to preserve cardiac hypertrophic mechanisms was the basis for the use of clenbuterol (a β-adrenergic agonist with potent anabolic properties) during LVAD unloading. (5). Despite the possibility of shorter duration of support offering the best clinical advantage, a
shorter duration may not be possible due to the scarcity of donors for heart transplant. It is possible that devices that achieve partial unloading may preserve compensatory hypertrophy and require a longer duration of support for recovery (25).

It has been previously proposed that adjuvant therapies may be the key to improving the recovery success of LVAD therapy (5), especially long-term therapy (28). Based on the results of this study, interesting targets for drug intervention would be CamKII, MEF2 and MSTN signaling (SMAD2), which worsened with longer LVAD support. Increased cytoplasmic CaMKII phosphorylation has been linked to dilated cardiomyopathy (40) and increased CaMKII levels during unloading have been previously reported in a rat model of heterotopic transplant (32). CamKII inhibition has been recently shown to improve ionotropy in cardiomyocytes from HF patients at the time of LVAD implantation (15). HDAC inhibitors have been proposed as potential therapeutic strategy for HF by limiting MEF2 activity - anti-hypertrophic effects (20) as well as anti-fibrotic effects (22) of HDAC inhibitors have been described in animal models, but use humans is in its infancy. MSTN inhibitors are currently being tested in clinical trials to treat Duchenne muscular dystrophy and have the potential of improving not only cardiac reverse remodeling but skeletal muscle cachexia associated to HF as well. The correlation between BMP1 levels and LV mass reversal suggests that BMP1 could be an interesting target to modulate MSTN. A novel finding in our study is the increase of stress sensor CRF1 in HF after LVAD. Modulation of urocortin receptors CRF1 and CRF2 may offer therapeutical benefits due to the cardioprotective role of these receptors modulating ERK, AKT and reducing apoptosis (19). CRF1 inhibition is currently being studied as a means to regulate ERK in depression and anxiety (39), and CRF2 inhibition has been proposed to prevent cardiac overload injury in mice (35).
Responsiveness to LVAD therapy, regardless of duration, may be influenced by the characteristics of the disease, which may make certain patients refractory to reverse remodeling. Improvement in ventricular function most frequently occurs in younger patients with shorter duration of heart failure (13,16). Multiple factors have been associated with myocardial recovery, including etiology of HF, preexisting fibrosis, pre-implant myocyte size and the duration of HF before implant (8,12,25). In the present study, none of these factors had a significant effect on molecular or clinical measurement change. Interestingly, in our study support by axial flow LVAD had a significant cofounding effect on LVAD duration being associated to P-SMAD2 reversal. In contrast, continuous flow LVADs have been previously associated with increased complications, and potentially lower rate of LV recovery in comparison with pulsatile devices; these changes may be attributed to the decrease in arterial pulsatility (11).

Limitations

One limitation of our study is the lack of serial tissue evaluation in the same patients, which would provide useful information about the dynamics of studied parameters during LVAD unloading. Sample size precluded analyzing the effects duration of heart failure or drug therapy and patients were not stratified based on these parameters, which could confound the interpretation of results. The small number of normal tissue samples may confound the relative measurements in pre and post LVAD groups. Importantly, 23 out of the 25 patients in our cohort were male and additional studies will be necessary to extrapolate the results from this study to women. As this study focused on molecular changes during LVAD support, the functional measurements in our study were limited to the echocardiography exam routinely performed in LVAD patients in our center. Additional studies investigating the effect of LVAD duration on functional measures, such as segment contractility, wall thickening during a cardiac cycle, and
LV compliance during ventricular loading may help define the ideal duration time for myocardial recovery.

Conclusions

The optimal duration of mechanical assistance in patients with HF, and the effect of LVAD duration on HF on biologic parameters of recovery has not been described. In the current study, we found that LVAD support is associated with CaMKIIδ activation, increased nuclear MEF2, sustained HDAC4 phosphorylation, ongoing structural protein dysregulation and sustained fibrosis and apoptosis. Our study suggests that a long duration of support may be detrimental for myocardial recovery, based on significantly worsened activation of CaMKIIδ, MEF2, MSTN signaling and apoptosis with longer duration of LVAD support. Further studies in larger patient populations are required to verify and define the exact duration of LVAD support for potential myocardial recovery, which can have important clinical implications for LVAD management, transplant consideration, and recovery protocols.

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Disclosures
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Table 1. Patient data and changes after LVAD support. BMI refers to body mass index; CHF, chronic heart failure; DCM, dilated cardiomyopathy, PCWP, pulmonary capillary wedge pressure; PWT, posterior wall thickness; LVEF, left ventricle ejection fraction; LVEDD, end-diastolic dimension of the left ventricle; IVS, interventricular septum; BNP, brain natriuretic peptide; MI, myocardial infarction; ACEI, angiotensin converting enzyme inhibitor; AM, amiodarone; BB, beta blocker; CB, Ca\(^{2+}\) blocker; DP, dipyridamole; DU, diuretic; ST, statin; W, warfarin. Values are means ± SE. Using the duration of LVAD as a categorical variable, we performed mixed-design ANOVA with “LVAD” as a within-subjects factor and “LVAD duration” as a between-subjects factor followed by Bonferroni post-hoc test to compare the effect of LVAD and duration of support on clinical measurements before and after LVAD. *p<0.05 vs. pre LVAD.

Figure 1. Summary of cellular signaling pathways involved in cardiac remodeling in dilated cardiomyopathy.

Figure 2. CamKII\(\delta\) and LVAD support. (A) Total and phosphorylated (P-) at Thr287 CaMKII\(\delta\) in normal (n=4) and failing hearts before (pre LVAD, n=15) and after (post LVAD, n=15) and representative blots, (B) Thr287 P-/total CaMKII\(\delta\) ratio level change in patients supported by LVAD for < or > median duration of support (270 days), (C) Total and P- at Thr287 cytoplasmic CaMKII\(\delta\), (D) total and P- at Thr305/306 cytoplasmic CaMKII\(\delta\), (E) cytoplasmic CaM, (F) total and P- at Thr287 nuclear CaMKII\(\delta\), (G) total and P- at Thr305/306 nuclear CaMKII\(\delta\), (H) nuclear CaM in normal (n=4), pre LVAD, (n=15) and post LVAD, n=15) heart samples, and representative blots. Molecular variables were compared using one-way ANOVA with post-hoc Bonferroni analysis. Simple linear regression analysis was used to determine the effect of LVAD time as a continuous variable in clinical and molecular measurements. T-testing was used to
compare effect on LVAD duration as a categorical variable in the change in molecular
measurement with LVAD. * p<0.05 vs. normal.

Figure 3. CamKIIδ-dependent signaling and LVAD support. Total and phosphorylated (P-)
(A) cytoplasmic HDAC 4, (B) HDAC5, (C) nuclear HDAC4 and (D) HDAC5; (E) cytoplasmic
MEF2 and (F) nuclear MEF2 in normal hearts (n=4) and samples from failing hearts before (pre
LVAD, n=15) and after (post LVAD, n=15) and representative blots, (G) nuclear MEF2 level
change in patients supported by LVAD for < or > median duration of support (270 days).
Molecular variables were compared using one-way ANOVA with post-hoc Bonferroni analysis.
Simple linear regression analysis was used to determine the effect of LVAD time as a
continuous variable in clinical and molecular measurements. T-testing was used to compare
effect on LVAD duration as a categorical variable in the change in molecular measurement with
LVAD. * p<0.05 vs. normal.

Figure 4. Myostatin and LVAD support. (A) Serum MSTN propeptide in normal donor serum
(n=4) and samples from DCM patients before (pre LVAD, n=10) and after (post LVAD, n=10)
LVAD implantation. (B) Cardiac MSTN propeptide levels in normal hearts (n=4) and samples
from failing hearts before (pre LVAD, n=15) and after (post LVAD, n=15), and representative
blots. (C) Cardiac MSTN propeptide level change in patients supported by LVAD for < or >
median duration of support (270 days). (D) Activin A, (E) ACTIIBr, and (F) BMP1 in normal
hearts (n=4) and samples from failing hearts before (pre LVAD, n=15) and after (post LVAD,
n=15), and representative blots. (G) BMP1 level change in patients supported by LVAD for < or
> median duration of support (270 days). (H) Graphical representation of the correlation
between left ventricle mass change and BMP1 protein level change with LVAD support. (E)
Cardiac total and phosphorylated SMAD2 ratio in normal hearts (n=4) and samples from failing
hearts before (pre LVAD, n=15) and after (post LVAD, n=15), and representative blots. (J)
Cardiac total and phosphorylated SMAD2 ratio change in patients supported by LVAD for < or > median duration of support (270 days). Molecular variables were compared using one-way ANOVA with post-hoc Bonferroni analysis. Simple linear regression analysis was used to determine the effect of LVAD time as a continuous variable in clinical and molecular measurements. T-testing was used to compare effect on LVAD duration as a categorical variable in the change in molecular measurement with LVAD. * p<0.05 vs. corresponding control (normal/short support).

**Figure 5. Stress and growth-related proteins and LVAD support.** (A) Total and phosphorylated ERK42, (B) total and phosphorylated ERK44, (C) total and phosphorylated MAPK p38, (D) total and phosphorylated AKT, (E) total and phosphorylated mTOR, (F) CRFR1, (G) CRFR2 in normal hearts (n=4) and samples from failing hearts before (pre LVAD, n=15) and after (post LVAD, n=15), and representative blots. Molecular variables were compared using one-way ANOVA with post-hoc Bonferroni analysis. Simple linear regression analysis was used to determine the effect of LVAD time as a continuous variable in clinical and molecular measurements. T-testing was used to compare effect on LVAD duration as a categorical variable in the change in molecular measurement with LVAD. * p<0.05 vs. normal # p<0.05 vs. pre LVAD.

**Figure 6. Structural Proteins, fibrosis and apoptosis and LVAD support.** (A) Actin, (B) myosin heavy chain, (C) desmin, (D) matrix metalloprotease 9 in normal hearts (n=4) and samples from failing hearts before (pre LVAD, n=15) and after (post LVAD, n=15), and representative blots. (E) Fibrosis levels in DCM (n=15) and after LVAD support (n=15), (F) representative images, (G) DNA fragmentation levels in DCM (n=15) and after LVAD support (n=15), in duplicate, (H) representative images, (I) DNA fragmentation change in patients
supported by LVAD for < or > median duration of support (270 days). Molecular variables were compared using one-way ANOVA with post-hoc Bonferroni analysis. Simple linear regression analysis was used to determine the effect of LVAD time as a continuous variable in clinical and molecular measurements. T-testing was used to compare effect on LVAD duration as a categorical variable in the change in molecular measurement with LVAD. * p<0.05 vs. normal.

**Figure 7. RNA sequencing and LVAD support.** Summary of RNA-seq results. Volcano plot representation of differential expression analysis of protein-coding genes in LV samples from DCM patients before (n=10) versus after LVAD support (n=10). Scattered points represent genes. The x-axis shows log2 fold changes in expression and the y-axis the statistical significance of a gene being differentially expressed. Genes with an absolute log2 fold change >1 are labeled. MSTN/SMAD-related signaling genes (*GADD45A*, *BMP2*, *DACT2*, *PLAU*, and *BMF*), calcium-sensing gene *HPCAL4* and C-type natriuretic peptide (*NPPC*) are highlighted in bold. The figure has been edited for readability, original image included in Supplementary Results.
Table 1. Patient Data and Changes after LVAD support

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* indicates a significant change.
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<tr>
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Gene expression:
- fetal/stress response genes:
  - MSTN
  - hypertrophy:
    - ECM remodeling (MMPs)
    - structural (desmin, SMA, actin)
- proliferation
- apoptosis

Figure 1
**Figure 2**

A. Total P-CaMKIIδ Thr287/CaMKIIδ

- **Normal**
- **Pre LVAD**
- **Post LVAD**

B. Total P-CaMKIIδ Thr287/CaMKIIδ change

- **Short support**
- **Long support**

C. Cyt. P-CaMKIIδ Thr287/CaMKIIδ

D. Cyt. P-CaMKIIδ Thr305/CaMKIIδ

E. Cyt. CaM

F. Nuc. P-CamKIIδ Thr287/CamKIIδ

G. Nuc. P-CamKIIδ Thr305/CamKIIδ

H. Nuc. CaM

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A  Cyt. P-HDAC4/HDAC4

B  Cyt. P-HDAC5/HDAC5

C  Nuc. P-HDAC4/HDAC4

D  Nuc. P-HDAC5/HDAC5

E  Cyt. MEF2

F  Nuc. MEF2

G  Nuc MEF2 change

Figure 3
Figure 4

A. Serum MSTN propeptide

B. Cardiac MSTN propeptide

C. Cardiac MSTN propeptide change

D. Activin A

E. ACTIIBR

F. BMP1

G. BMP1 change

H. LV mass change with LVAD

I. P-SMAD2/SMAD2

J. P-SMAD2/SMAD2 change
Figure 5
Figure 6

A. Actin

B. SM MHC

C. Desmin

D. MMP9

E. Fibrosis

F. TUNEL positive cells/1000 cells

G. Apoptosis

H. Apoptosis change
Figure 7