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Therapeutic Molecular Phenotype of β-blocker Associated Reverse-remodeling in Nonischemic Dilated Cardiomyopathy

David Kao, MD[1], **Brian Lowes, MD PhD**[2], **Edward Gilbert, MD**[3], **Wayne Minobe**[1], **L Elaine Epperson, PhD**[1], **Leslie Meyer**[1], **Debra Ferguson, ANP-BC**[1], **Kirk Volkman, APRN**[3], **Ronald Zolty, MD PhD**[4], **Alastair Robertson, PhD**[1], **Douglas Borg, MD**[5], **Robert Quaife, MD**[1], **Michael Bristow, MD PhD**[1]

[1]University of Colorado School of Medicine, Aurora, CO.

[2]University of Nebraska Medical Center, Omaha, NE.

[3]University of Utah, Salt Lake City, UT.

[4]Albert Einstein College of Medicine, Bronx, NY.

[5]Heart Clinic of Arkansas, Little Rock, AR

Abstract

Background: When β-blockers produce reverse-remodeling in idiopathic dilated cardiomyopathy (IDC) they partially reverse changes in fetal-adult/contractile protein, natriuretic peptide, SR-Ca2+-ATPase gene "program" constituents. The objective of the current study was to further test the hypothesis that reverse-remodeling is associated with favorable changes in myocardial gene expression by measuring additional contractile, signaling and metabolic genes that exhibit a fetal/adult expression predominance, are thyroid hormone-responsive, and/or are regulated by β_1-adrenergic receptor signaling. A secondary objective was to identify which of these putative regulatory networks is most closely associated with observed changes.

Methods and Results: Forty-seven IDC patients (LVEF 0.24±0.09) were randomized to the adrenergic-receptor blockers metoprolol (β_1-selective), metoprolol+doxazosin (β_1/α_1), or carvedilol ($\beta_1/\beta_2/\alpha_1$). Serial radionuclide ventriculography and endomyocardial biopsies were performed at baseline, 3, and 12 months. Expression of 50 mRNA gene products was measured by quantitative PCR. Thirty-one patients achieved LVEF reverse-remodeling response defined as improvement by ≥0.08 at 12 months or by ≥0.05 at 3 months (ΔLVEF=0.21±0.10). Changes in gene expression in Responders vs. Nonresponders were decreases in *NPPA* and *NPPB* and increases in *MYH6*, *ATP2A2*, *PLN*, *RYR2*,*ADRA1A*, *ADRB1*, *MYL3*, *PDFKM*, *PDHX*, and *CPT1B*. All except *PDHX* involved increase in adult or decrease in fetal cardiac genes, but 100% were concordant with changes predicted by inhibition of β_1-adrenergic signaling.

Conclusions: In addition to known gene expression changes, additional calcium-handling, sarcomeric, adrenergic signaling, and metabolic genes were associated with reverse-remodeling.

Corresponding author: Michael Bristow, 12700 E 19th Ave Box B-139, Aurora, CO 80045, Michael.Bristow@ucdenver.edu, Phone: 303-724-5453, Fax: 303-724-5450.

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The pattern suggests a fetal-adult paradigm but may be due to reversal of gene expression controlled by a 'β_1-adrenergic receptor gene network.'

Trial Registration: NCT01798992 (www.clinicaltrials.gov/ct2/show/NCT01798992)

Keywords

myocardial gene expression; beta-blocker; dilated cardiomyopathy; heart failure

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[155]Physiological and pathological control of gene expression; [93]Receptor pharmacology; [110]Congestive heart failure; [115]Remodeling

INTRODUCTION

Idiopathic dilated cardiomyopathy (IDC) is a common type of nonischemic dilated cardiomyopathy (DCM) for which no cause is readily apparent.[1] The main phenotypic feature of IDC and other types of Heart Failure with reduced Ejection Fraction (HFrEF) is ventricular chamber remodeling characterized by contractile dysfunction and "eccentric" pathologic hypertrophy. These processes are commonly detected and quantified by measurement of left ventricular (LV) volumes and the derived ejection fraction (EF), which relates a measure of systolic function (stroke volume) to the degree of eccentric hypertrophy (end diastolic volume, EDV). IDC in humans is associated with myocardial gene expression changes affecting contractile function and hypertrophy.[2,3] Among these are up-regulation in mRNA and protein abundances of β-myosin heavy chain (*MYH7*) and atrial natriuretic peptide (*NPPA*) with down-regulation of α-myosin heavy chain (*MYH6*) and sarcoplasmic reticulum calcium-ATPase 2 (*ATP2A2*), a pattern also observed during fetal cardiac development.[2,3] Reversal of these pathologic changes is associated with ventricular reverse-remodeling in response to both β_1-selective and nonselective β-adrenergic receptor (AR) antagonists,[3] suggesting that reversal of abnormalities in gene expression driven by chronic β_1-AR stimulation may mediate salutary effects of β-blockers in IDC and potentially other forms of HFrEF.

Relationships between β_1-adrenergic signaling, changes in contractile- and hypertrophy-related gene expression, progression of LV dysfunction and reversal of molecular and structural remodeling by blockade of β_1-ARs are incompletely understood. Analysis of gene expression in septal endomyocardial biopsies obtained by right heart catheterization in the presence of β-blocker-associated reverse-remodeling constitutes a human model of gene function associated with changes in ventricular myocardial phenotype. We previously reported effects of β-blockers on human myocardial remodeling and gene expression using an early-generation method of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) that could measure only a small number of mRNAs.[3] That work was necessarily narrowly focused, and other gene categories potentially important in therapeutic response to β-blockers were not investigated. New tools using small amounts of RNA including improvements in qPCR methods and microarrays[4] have greatly increased the potential yield

of investigations where serial measurements of gene expression are coupled to therapeutic modulation of organ-specific phenotype.

We present here the β-blocker Effect On Remodeling and Gene Expression Trial (BORG, NCT01798992), which combined gene expression analysis of endomyocardial biopsy specimens with phenotypic measurements of LV structure and function. BORG is a next-generation longitudinal study of myocardial gene expression and reverse-remodeling in IDC patients treated with 3 different regimens of AR antagonists that have in common blockade of β_1-AR. BORG investigated relationships between myocardial gene expression and cardiac phenotypic change, the contribution of α_1- and β_2-AR blockade, and changes in expression of gene families beyond the current cardiac fetal/adult gene program paradigm. The primary hypothesis was that ventricular reverse-remodeling associated with β_1-AR blockade is driven by changes in contractile- or hypertrophy-modifying myocardial gene expression that either precedes or occurs contemporaneously with reverse-remodeling. Specifically, we hypothesized that candidate genes involved in AR signaling, renin-angiotensin and endothelin systems, cytokine signaling, muscle contraction, calcium handling, metabolism, and gene transcription would exhibit unique changes comprising a therapeutic molecular phenotype associated with ventricular reverse-remodeling and that observed changes would fit into a fetal/adult[3], β_1-AR driven[5,6] or thyroid hormone-responsive pattern.[7] To test this hypothesis, we quantified candidate gene mRNA expression using qPCR and microarray analysis in the context of changes in LV structure and function in response to β-blocker therapy.

METHODS

The primary outcome was a positive LVEF reverse-remodeling response defined as an improvement ≥8 EF units at 12 months or if not available, an improvement ≥5 units at 3 months (last-observation-carried-forward or LOCF). The LVEF definition of β-blocker response was based on previous observations of improved LVEF with β-blockers compared to placebo.[3,8] The 3-month LVEF response cutoff was based on previously demonstrated favorable changes in fetal gene program components associated with an improvement ≥5 EF units after a longer treatment interval of 6 months.[3] The 12-month cutoff was defined based on a blinded, prospective analysis of mean LVEF improvement at 12 months among those with improvement ≥5 EF units at 3 months. Nonresponse was defined as not meeting the positive response criteria or the occurrence of heart transplant, left ventricular assist device placement or death.

Expression of 50 candidate mRNA gene products and 2 reference genes (Table 1) was quantified by RT-qPCR. Gene expression changes associated with pathologic LV remodeling were identified by comparing differences in gene expression between IDC and nonfailing control patients. Changes associated with reverse-remodeling were identified by comparing gene expression at LOCF to baseline gene expression in patients with a positive LVEF response (“Responders”) to patients with LVEF nonresponse (“Nonresponders”).[3] Analysis of differences in gene expression between the 3 AR-blocking groups was a secondary objective and will be reported separately.

Study design

This study was conducted between 2000 and 2008 at the Universities of Colorado and Utah according to the Declaration of Helsinki and included a Data Safety Monitoring Board. The study was approved by institutional review boards at both sites, and all subjects gave written informed consent. Patients were eligible for enrollment if they had IDC and New York Heart Association (NYHA) Class II-IV symptoms with an LVEF ≤40%, were ≥18 years old, had angiographically-confirmed unobstructed coronary arteries, and were receiving conventional medical HF therapy except β-blockers for ≥3 weeks prior to enrollment. Exclusion criteria included HF due to valvular disease; thyroid disease; obstructive or hypertrophic cardiomyopathy; pericardial disease; amyloidosis; myocarditis; heart transplant candidacy; decompensated HF; ongoing treatment with nondihydropyridine calcium channel blockers, theophylline, tricyclic antidepressants, monoamine oxidase inhibitors, β-agonists, β-blockers, or inotropes; life expectancy<2 years; active substance abuse; recently fired implantable cardiac defibrillator; bradycardia; uncontrolled insulin-dependent diabetes; high-degree atrioventricular block; or history of noncompliance.

Patients were randomized in an unblinded fashion to commercially available formulations of carvedilol (COREG®), metoprolol succinate (TOPROL-XL®), or metoprolol succinate +doxazosin mesylate (CARDURA®). The starting dose of carvedilol, a $\beta_1/\beta_2/\alpha_1$-AR blocking agent, was 3.125 mg twice daily with targets of 25 mg (patients<85 kg) or 50 mg (patients≥85 kg) twice daily.[9] The starting dose of metoprolol succinate, a β_1-AR selective blocking agent, was 12.5 mg daily with a target of 200 mg daily.[10] Doxazosin mesylate, an α_1-AR selective blocking agent, was started at 1 mg daily with a target of 8 mg daily.[11] All medications were up-titrated weekly until target doses were met or limiting side effects developed.

Endomyocardial biopsy may be considered clinically in IDC patients to rule out causes of myocardial disease that might affect prognosis or therapy.[1,12] Patients underwent right heart catheterization via right internal jugular vein with RV endomyocardial biopsy from the distal septum at baseline, 3, and 12 months where the 3 and 12 month biopsies were used for RNA extraction for research purposes only. Biopsies were performed on all IDC patients by personnel experienced in performing RV biopsies in non-transplant patients under echocardiographic guidance, and 15–25 mg of tissue was removed. Biopsies were also taken from 5 potential nonfailing control patients with LVEF≥45% who were studied for other indications to rule out myocardial disease. Histologic analysis of baseline biopsies reported hypertrophy in 71%, increased interstitial fibrosis in 39%, and evidence of inflammation/mononuclear infiltrate in 11%. One potential control was excluded due to giant cell myocarditis on histologic examination, whereas the 4 other potential control biopsies had no evidence of hypertrophy, fibrosis, or inflammation and were used in the study. LVEF was measured within days of each biopsy by radionuclide ventriculography (see Supplement). RNA was extracted from all IDC patient and nonfailing control biopsy samples, cDNA was synthesized, and expression of 50 candidate genes (Table 1) was quantified by RT-qPCR using threshold cycle (C_t) detection (see Supplement for methods and primer sequences). Gene expression was also measured by cDNA hybridization to the Affymetrix HG-U133 Plus 2.0 Gene Chip (see Supplement).

Statistical Analysis

Clinical and gene expression data were imported into the R statistical package (version 3.0.2, R Foundation for Statistical Computing, Vienna Austria). Patients were aggregated by LVEF response status, and clinical differences between responder groups were assessed using Fisher's exact and Welch's t-test for categorical and continuous variables. Marked skew was noted in some C_t distributions, and outliers were identified and removed using a median absolute deviation threshold>3.5, congruous with an alpha of 0.05 using parametric statistical analysis (276 of 13100 values, 2.1%).[13] If a subject's baseline sample was excluded or unavailable, follow-up samples were excluded. Changes in gene expression from baseline to LOCF (month 12 values or if not available, month 3 values) and with treatment were approximated using the $\Delta\Delta C_t$ method with *GAPDH* as the reference gene.[14] Changes in gene expression were compared between Responders and Nonresponders using the non-parametric Wilcoxon rank-sum test. Significance of changes in gene expression from baseline was determined using the non-parametric paired Wilcoxon signed-rank test in Responders and Nonresponders separately. Fold changes and aggregate statistics were calculated using normalized C_t differences ($2^{-\Delta\Delta Ct}$). All tests were two-tailed, and a $p<0.05$ was considered significant.

Comparisons were repeated using the arithmetic mean of *GAPDH* and *18S rRNA* expression to determine effect of normalization strategy on results. Microarray data were normalized by log-scale robust multi-array analysis.[15] Cognate microarray data corresponding to the 50 RT-qPCR genes were also analyzed, and changes in expression from baseline were compared between Responders and Nonresponders using the Wilcoxon rank-sum test. A $p<0.05$ by microarray was considered confirmatory of a significant RT-qPCR finding.

RESULTS

Outcomes of Enrolled Patients

A total of 63 IDC patients met screening criteria and gave written informed consent (Figure 1). Of these, 6 subjects withdrew prior to baseline studies for personal or administrative reasons, 2 had normalization of a reduced LVEF obtained on a previous examination, and central venous access could not be obtained in 1 leaving 54 subjects who underwent baseline studies. In 2 subjects, radionuclide ventriculography revealed their LVEFs had normalized since screening, and their biopsy samples were analyzed as nonfailing controls. Of the randomized 52 subjects, 47 returned for 3 month studies and 40 returned for 12 month studies. Therefore, 47 patients had ≥1 follow-up biopsy and LVEF measurement after baseline and were included in all subsequent analyses. There were no complications from endomyocardial biopsy or right heart catheterization.

In total 39 of 47 (83.0%) patients achieved target β-blocker dose including 16 of 16 carvedilol patients(mean daily dose 75±24 mg), 13 of 17 (76.5%) metoprolol patients (mean daily dose 163±60 mg), and 10 of 14 (71.4%) metoprolol+doxazosin patients(mean daily metoprolol dose 171±51 mg). Target doxazosin dose was reached in 10 of 14 patients (71.4%, mean daily dose 6±3 mg). Of the 5 patients not analyzed, 1 subject had inadequate tissue for RT-qPCR at the only follow-up visit, 2 relocated, and 2 were withdrawn for

administrative reasons. One patient in the carvedilol arm died suddenly 10 months after randomization, and 1 patient underwent ventricular assist device placement after 16 months. There were no heart transplants during the study. A total of 8 patients (4 Responders, 4 Nonresponders) had 3 to 12 months LOCF imputation.

Baseline characteristics of IDC vs. nonfailing control subjects and LVEF Responders vs. Nonresponders are shown in Tables 2a–b. Significant differences between IDC and nonfailing subjects were limited to LVEF, RVEF, LV dimensions, NYHA class, and cardiac index. In total, 31 of 47 (66.0%) of patients met criteria for LVEF response. Baseline clinical features significantly associated with LVEF response included shorter duration of HF, higher estimated creatinine clearance, and narrower QRS duration. β-blocker doses were not significantly different between Responders and Nonresponders in any treatment arm (carvedilol: 78±23 vs. 71±27 mg, p=0.64; metoprolol: 179±50 vs 125±41 mg, p=0.1: metoprolol+doxazosin: 165±58 vs. 188±25 mg, p=0.29).

Changes in cardiac function, volume, and hemodynamic parameters are compared between Responders and Nonresponders in Table 3. Responders demonstrated significant improvement in LVEF (ΔLVEF=21.2±9.8 vs. 1.4±4.9 EF units in Nonresponders, p<0.001) and LV size (ΔLV EDV, −82±60 vs. 16±58 ml in Nonresponders, p<0.001). Heart rate decreased significantly more in Responders compared with Nonresponders (−18.2±20.6 vs. −4.7±13.3 bpm, p<0.001), as did pulmonary artery pressure (−4.6±8.4 vs. 1.7±8.8 mm Hg, p<0.05). Although RVEF improved significantly in Responders (27.7±8.7 to 37.0±7.6 EF units, p<0.001) and not in Nonresponders (27.2±9.7 to 32.0±11.6 EF units, p=0.14), the respective changes were not significantly different (p=0.27). Systolic blood pressure and cardiac index also increased significantly in Responders only compared to baseline, but differences between responder groups were not significant.

Gene Expression Changes

Expression of the 50 candidate genes normalized to *GAPDH* at baseline in IDC patients is compared with control patients in Figure 2a, b, c. Compared to controls, 11 of 50 (22.0%) genes were differentially expressed in IDC patients. *ADRB1*, *ATP2A2*, *MYH6* and *ACTC1* were expressed at significantly lower levels in IDC patients, whereas *MYL2*, *HK2*, *PDHX*, *CTF1*, *TNNI3*, *NPPA*, and *NPPB* were expressed at significantly higher levels. The *ACTC1*/*ACTA1* ratio was significantly lower in IDC patients compared to nonfailing controls (0.77±0.09 vs. 1.76±0.5, p=0.024), whereas *MYH6*/*MYH7* (p=0.06) and *ATP2A2*/*PLN* (p=0.28) ratios were not significantly different.

Fold changes in myocardial gene expression from baseline by responder status are shown in Figure 3a, b, c. Expression of 13 of 50 (26.0%) genes (*ACTA1, TNNC, TNNI3, IL6, GNAI2, GNAS*, *SLC8A1*, *SLC9A1*, *MYL2*, *ACTC1*, *HK2*, *CTF1,* and *CSRP3)* decreased significantly AND 4 of 50 (8.0%) genes (*TNNT2, CANX, PDK4, Tr-α1*) increased significantly in Responders with no differences vs. changes in Nonresponders. Expression of 11 of 50 (22.0%) genes (*ADRB1, ADRB2, ADRA1A*, *ATP2A2*, *PLN*, *RYR2*, *MYH6*, *MYL3*, *CPT1B*, *PDHX,* and *PFKM*) increased significantly or decreased less in Responders vs. Nonresponders, whereas expression of 2 (4.0%) genes (*NPPA* and *NPPB*) decreased significantly in Responders vs. Nonresponders. *MYH6*/*MYH7* (1.96±0.32 vs. 0.78±0.07,

p<0.001) and *ACTC1*/*ACTA1* (1.91±0.32 vs. 0.87±0.11,p<0.05) ratios also increased in Responders, whereas change in the *ATP2A2*/*PLN* ratio was not significantly different (p=0.37) between responder groups. When 3 (N=47) and 12 month (N=39) samples were considered separately, *PFKM* and *RYR2* were significantly different in Responders vs. Nonresponders at 3 months only, *ADRB1*, *ADRB2*, *ATP2A2*, *PLN*, *MYH6*, and *MYL2* were significantly different at 12 months only, and *NPPA*, *NPPB*, and *MYL3* were significantly different at both time points. No genes were significant at either 3 or 12 months that were not significant in LOCF analysis. Neither *GAPDH* nor *18S rRNA* changed significantly in analysis of nonnormalized Ct data (p>0.4, p>0.3, respectively). However expression of *18S rRNA* decreased significantly relative to *GAPDH* on all 4 qPCR plates in Responders (fold change 0.47–0.59, p<0.01) but not in Nonresponders (p>0.14). All genes with significant differences between Responders and Nonresponders in serial gene expression normalized to *GAPDH* alone remained significant when normalized to the composite of *GAPDH* and *18s rRNA*. The ratio of *MYH6*/*MYH7* was also significantly higher in Responders vs. Nonresponders normalized to the composite of *GAPDH* and *18S rRNA* (2.00±0.30 vs. 0.9±0.11, p<0.01), whereas the *ACTC1*/*ACTA1* ratio was higher in Responders but not statistically significant (1.98±0.51 vs. 1.00±0.20, p=0.10).

Genes that demonstrated differential expression changes between Responders and Nonresponders by RT-qPCR were also tested by microarray. Of these, 8 of 13 genes (*MYH6*, *MYL3*, *PLN*, *RYR2*, *NPPA*, *NPPB*, and *PFKM*) were also significantly differentially expressed on microarray with the same directionality (p<0.05). Increases in *MYH6*/*MYH7* and *ACTC1*/*ACTA1* ratios were also significant (p<0.01) by microarray in Responders compared to Nonresponders. Changes in *ADRB1*, *ATP2A2*, *ADRA1A, CPT1B,* and *PDHX* had the same directionality by microarray analysis and RT-qPCR, but differences between responder groups were not significant.

DISCUSSION

These data provide the most comprehensive longitudinal analysis reported to date of myocardial gene expression associated with reverse-remodeling in DCM patients. Because the intraventricular septum is shared between the ventricles and forms interdependent anatomic relationships with both the LV and RV free walls,[16] molecular changes relative to LV structure and function will be detected by septal endomyocardial biopsy.[3] In the current study Responders exhibited a robust improvement of 21±10 EF units, whereas changes in RVEF and hemodynamic parameters were not significantly different between Responders and Nonresponders. Therefore, it is likely that the observed changes in gene expression selectively present in Responders reflect intrinsic biologic changes in the LV chamber as opposed to changes in RV function, heart rate or LV loading conditions. In addition, the study design comparing Responders to Nonresponders who were given equivalent β_1-AR blocking target doses[3] at similar levels in Responders and Nonresponders minimizes the degree of β_1-blockade as a mechanism for the observed differences. The present study provides insight into gene expression changes associated with LV reverse-remodeling with improvement in systolic function or regression of pathologic hypertrophy as well as myocardial response to β-blocker therapy irrespective of reverse-remodeling.

Gene Expression Changes in β-blocker Treatment Associated with Reverse-remodeling

The observed changes indicate increased expression in genes affecting contractile function, calcium handling, energy substrate utilization and adrenergic signaling plus decreases in natriuretic peptides, which are biomarkers of failing or hypertrophied myocardium. We reported previously that when β-blocking agents produce reverse-remodeling in IDC, they effect a partial reversal of HF-associated changes in expression of genes encoding β_1-AR (*ADRB1*),[17] contractile proteins (*MYH6* and *MYH6/MYH7* ratio),[3] natriuretic peptides (*NPPA*),[3] and sarcoplasmic reticulum calcium-ATPase 2 (*ATP2A2*).[3] The results of BORG confirm those findings and extend the observations to increases in expression of additional contractile (*ACTC1*/*ACTA1* ratio, *MYL3*) and calcium-handling genes (*PLN*, *RYR2*).

We also report changes in additional categories of genes associated with reverse-remodeling including the metabolic pathway genes *CPT1B* and *PFKM* and the α_1-AR gene *ADRA1A*. Utilization of both fatty acids and glucose as energy sources is decreased in advanced HF[18] limiting ATP synthesis. Coordinated up-regulation of both *PFKM* and *CPT1B* has been demonstrated in response to β-blockade suggesting a return to an adult metabolic gene expression pattern.[19] If accompanied by cognate changes in protein abundance increased *CPT1B* should increase fatty acid metabolism,[20] whereas increased *PFKM* should enhance glucose metabolism. Therefore, up-regulation of both free fatty acid and glucose metabolism occur to achieve higher energy production as LV function improves.

If mRNA abundance changes observed exclusively in Responders were translated to protein expression and activity, LV contractile function would be expected to increase due to the improved *MYH6*/*MYH7* ratio and increased expression of *MYH6*,[21] *MYL3*,[22] *ATP2A2*,[23] *RYR2*,[24] *ADRA1A*,[25] and *ADRB1*.[26] In addition, decreases in *MYH7*/*MYH6*[27] and *ACTA1*/*ACTC1*[28] ratios and reduction in *18S rRNA*[29] should be anti-hypertrophic. Up-regulation in unphosphorylated *PLN* might be negatively inotropic,[30] but increased *ADRB1* expression would increase phosphorylated *PLN* potentially offsetting the impact of higher *PLN* expression.[31] Decreases in *NPPA* and *NPPB* might be expected to be hypertrophic,[32] but a previous study also observed that *NPPA* decreased in both Responders and Nonresponders, presumably as intracardiac filling pressures fell,[3] and circulating B-type natriuretic peptide has been observed to decrease in association with reverse-remodeling.[33] These data suggest that β_1-AR blockade, common to all treatment arms, may effect reverse-remodeling by increasing expression of contractile, calcium-handling/-regulating, adrenergic receptor, and metabolic proteins that either improve LV contractile function or participate in its metabolic support.

Of 11 genes that exhibited altered baseline expression in IDC compared to nonfailing controls, 5 (*ADRB1*, *MYH6*, *ATP2A2*, *NPPA*, *NPPB*) were changed in the direction of control values (“normalized”) only in Responders, 2 (*TNNI3* and *MYL2*) normalized in Responders without a significant difference vs. Nonresponders, and 3 (*HK2, CTF1, PDHX*) normalized in both Responders and Nonresponders. These data suggest that β-blocker therapy effects reverse-remodeling by partially normalizing some gene expression changes fundamentally associated with the DCM phenotype.

Many β-blocker associated gene expression changes in Responders vs. Nonresponders represent a partial reversal of the cardiac fetal/adult gene program,[3,34,35] but they may also be consistent with alternative regulatory mechanisms including inhibition of β_1-AR signaling[5,6,36] and/or enhanced thyroid hormone receptor (TR-α) activity (Table 4).[7] We previously reported changes in *TR-α1* and *TR-α2* expression in IDC LVs consistent with hypothyroidism at the TR-α level. Furthermore, myocardial expression of *TR-α1* is positively correlated with *MYH6* expression and negatively correlated with *ANP*, whereas *TR-α2* expression is negatively correlated with *MYH6* and positively with *ANP* expression, suggesting that TR-α expression may influence fetal/adult gene program regulation. In the current study changes in expression of either TR-α isoform were not different between responder groups, although *TR-α1* expression increased significantly in Responders only, suggesting a TR-α effect was not completely ruled out.

As shown in Table 4, changes in gene expression associated with LV reverse-remodeling were 100% concordant with changes expected from interruption of chronic β_1-AR stimulation[5,36] or cardiac β_1-AR overexpression,[6] both of which influence the fetal/adult gene program via the Ca2+/calmodulin-dependent kinase pathway.[5] Thus the data are most consistent with a primary effect of a β_1-blockade of a "β_1-AR gene network" in Responders overlapping with effects expected for TR-α responsive and fetal/adult developmental gene expression patterns. The expansion of known genes whose expression changes in the setting of β-blocker therapy for IDC may provide additional insight into primary regulatory pathways involved in LV reverse-remodeling. Exploration of these findings may identify factors that determine whether β-blockade produces changes in myocardial gene expression that promote reverse-remodeling in a given individual, i.e. why the β_1-AR network is affected by β_1-blockade in reverse-remodeling patients only.

Gene expression changes associated with β-blocker therapy

Gene expression changes that occur in both Responders and Nonresponders are the likely result of pharmacologic effects of β_1-AR blockade as opposed to being secondary to ventricular reverse-remodeling. Expression of genes encoding contractile (*ACTC1*), metabolic (*HK2*, *PDHX*), signaling (*CSRP3, GNAI2, GNAS*, *SLC9A1*), and calcium-handling proteins (*SLC8A1*) all decreased significantly in both Responders and Nonresponders. In some cases, these changes represent partial reversal of pathologic changes associated with IDC even when not associated with improvement in LVEF. For example, *SLC8A1* has been shown to be up-regulated in the failing human heart, [37] and inhibition of *SLC8A1* has been associated with reduced arrhythmia in experimental models of DCM, suggesting potential for improved survival even without reverse-remodeling.[38]

Comparison of Expression Changes in Responders to Previous Studies of Reverse-Remodeling in Human Dilated Cardiomyopathies

Previous studies using cardiac resynchronization therapy[39–41] or β-blockers[3,17,42–44] to effect reverse-remodeling have reported similar protein or mRNA gene expression changes in *ADRB1*+*ADRB2*,[17,42] *ADRB1*,[39] *MYH6*,[3,40,41,43] *MYH6*/*MYH7*, [3,40,41,43] *ATP2A2*,[3,41,44] *PLN*,[40,41,44] *NPPA*[3] and *NPPB*.[41] Responder-specific gene expression

changes in *RYR2*, *ADRA1A*, *MYL3*, *PDKFM*, *PDHX*, *CPT1B*, and *ACTA1/ACTC1* have not been previously reported in reverse-remodeling of human dilated cardiomyopathies.

Limitations

Analysis was limited to gene expression changes, which may not be directly translated to changes in functional protein abundance. Because of the quantity of RNA required to perform the mRNA analyses reported in this study as well as additional microRNA and RNAseq measurements quantification of protein expression or post-translational modification was not feasible and was not a prespecified aim of the present study. Conclusions regarding significance of gene expression changes may vary according to assay and normalization standard. *GAPDH* was selected at the time of trial design based on its widespread use as a reference gene, its mid-range expression level and lack of common regulation, but use of *GAPDH* as a normalization standard has subsequently been questioned.[45] Ribosomal *18S rRNA* was quantified but is not generally used alone as a reference standard due to its high abundance and the unpredictable balance of rRNA and mRNA in different tissues.[46] We repeated RT-qPCR analysis using a composite of *GAPDH* and *18S rRNA*, and all genes associated with LV response remained significant. We used microarray analysis as an additional confirmatory assay although microarray analysis uses a distinct normalization strategy, and the importance of differences in statistical significance between RT-qPCR and microarray findings is unclear.

Carvedilol is a “biased ligand” for both β_1[3,47–50] - and β_2-ARs, meaning that it can G-protein independently activate MAP kinase ERK1,2 pathways via β-arrestin signaling as it produces receptor blockade. Metoprolol has also been shown to be a biased ligand for β_1-ARs, in a pathway that appears to be different from carvedilol. In a previous study we found absolutely no differences between metoprolol vs. carvedilol for the 6 mRNAs measure by quantitative PCR. In the current study there were some differences between the carvedilol and the combined metoprolol groups, but the statistical results of the metoprolol groups remained unchanged when combined with the carvedilol group. We will be subsequently reporting a complete analysis of gene expression between groups, where the consequences of differences in biased ligand signaling in the carvedilol vs. metoprolol groups may become apparent.

CONCLUSIONS

LV reverse-remodeling in IDC patients receiving β-blocker therapy is associated with a distinct therapeutic molecular phenotype consisting of changes in expression of genes involved in AR signaling, contractile protein function, calcium handling, and metabolism. These changes are directionally opposite of previously described gene expression effects of chronic β_1-AR stimulation,[5,36] or transgenic myocardial overexpression,[6] which collectively constitute a β_1-AR gene network. The biologic effects of this gene network ultimately result in contractile dysfunction, hypertrophy and adverse effects on myocardial metabolism despite their positive inotropic acute effects.[8,51] The explanation for this biologic dichotomy appears to be distinct differences in signaling with chronic gene expression changes mediated by protein kinase A (PKA)-independent pathways[5] as opposed to PKA-dependent

acute effects. Changes in expression of these gene families may provide opportunities for directed pharmacologic therapy, and further study is warranted to identify key regulatory nodes responsible for β-blocker therapy inhibiting the β_1-AR gene network in some IDC patients (Responders) and not in others..

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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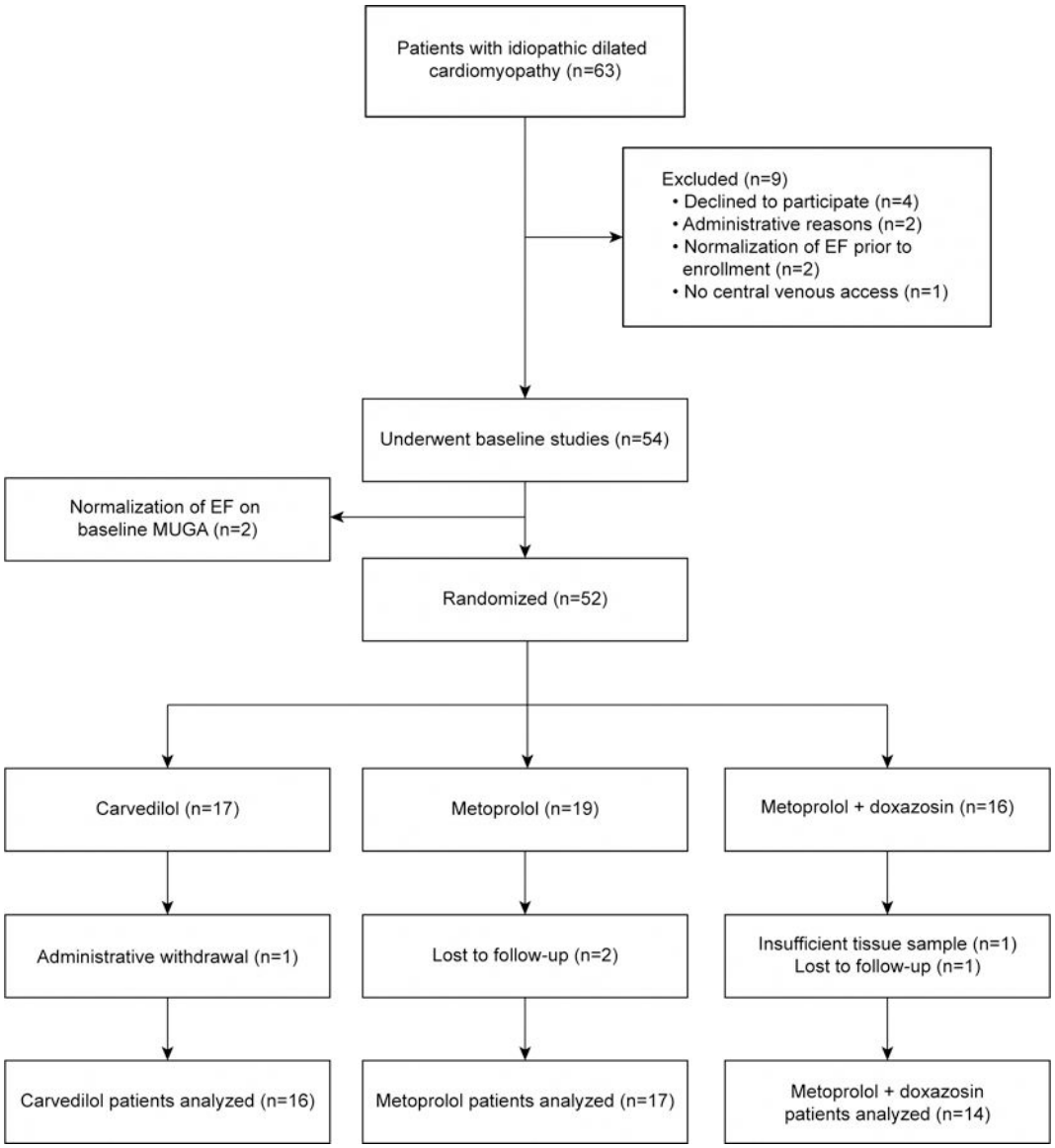

Figure 1 –.
Screening, enrollment and randomization

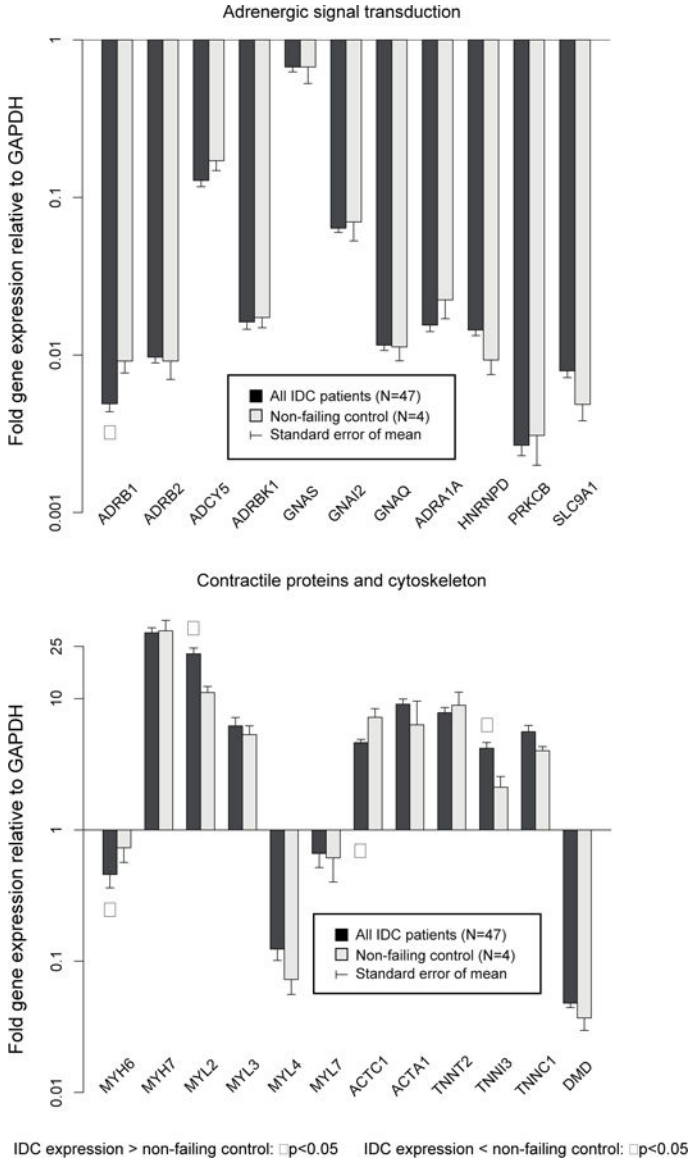

Figure 2a –. Baseline gene expression normalized to GAPDH: adrenergic signaling and contractile/cytoskeleton proteins in IDC (N=47) vs. controls (N=4).

ADRB1, *MYH6*, and *ACTC1* expressed at lower levels in IDC than controls, whereas *MYH2*, and *TNNI3* were expressed at higher levels in IDC patients.

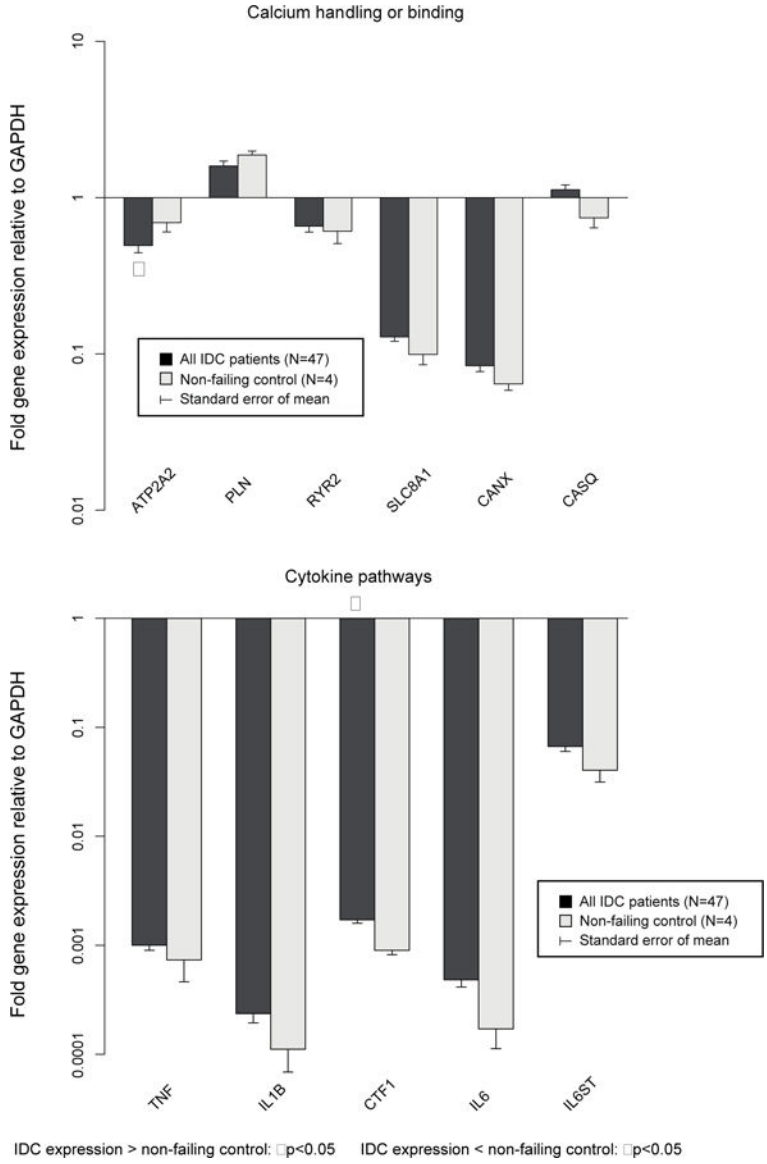

Figure 2b –. Baseline gene expression normalized to GAPDH: calcium handling and cytokine pathways in IDC (N=47) vs. controls (N=4).

ATP2A2 was expressed at lower levels in IDC than controls, whereas *CTF1* was expressed at higher levels in IDC patients.

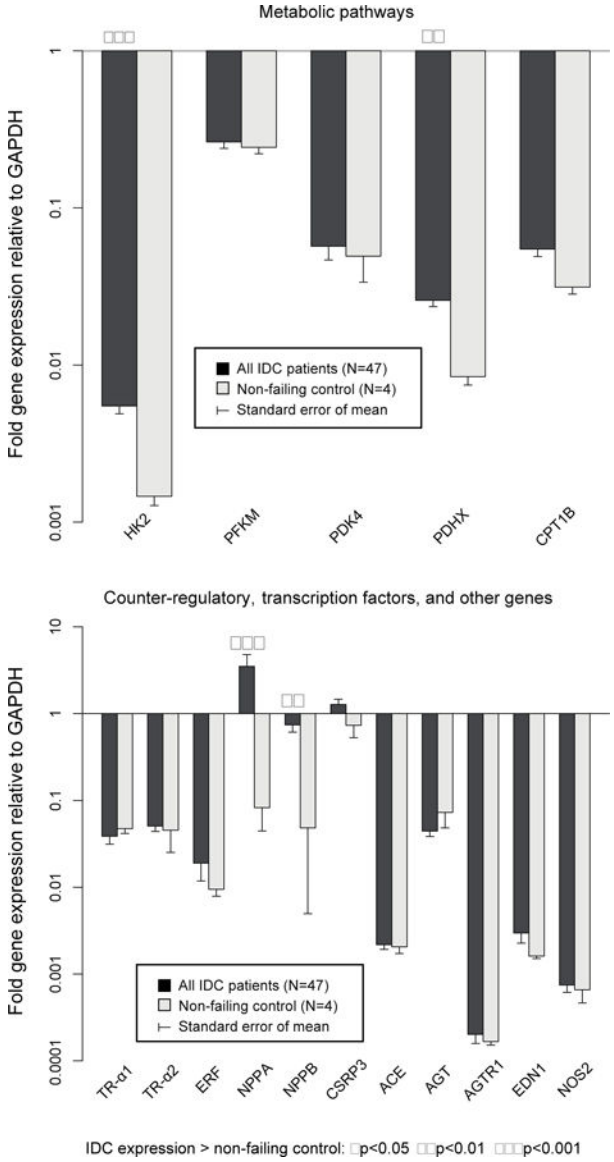

Figure 2c –. Baseline gene expression normalized to GAPDH: metabolic pathways and counter-regulatory, transcription factors, and other genes in IDC patients (N=47) vs. controls (N=4).
HK2, PDHX, NPPA and *NPPB* were expressed at higher levels in IDC patients.

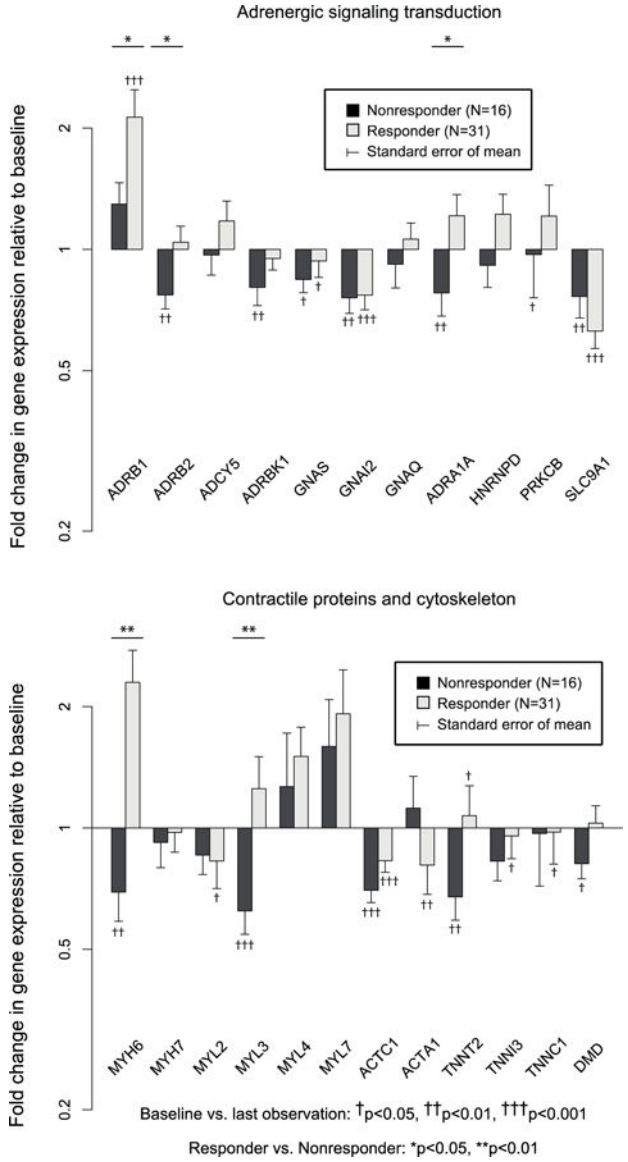

Figure 3a –. Fold change in gene expression: adrenergic signal transduction and contractile/ cytoskeleton proteins in Responders (N=31) vs. Nonresponders (N=16).

ADRB1, ADRB2, ADRA1A, MYH6, and *MYL3* were upregulated in Responders compared with Nonresponders.

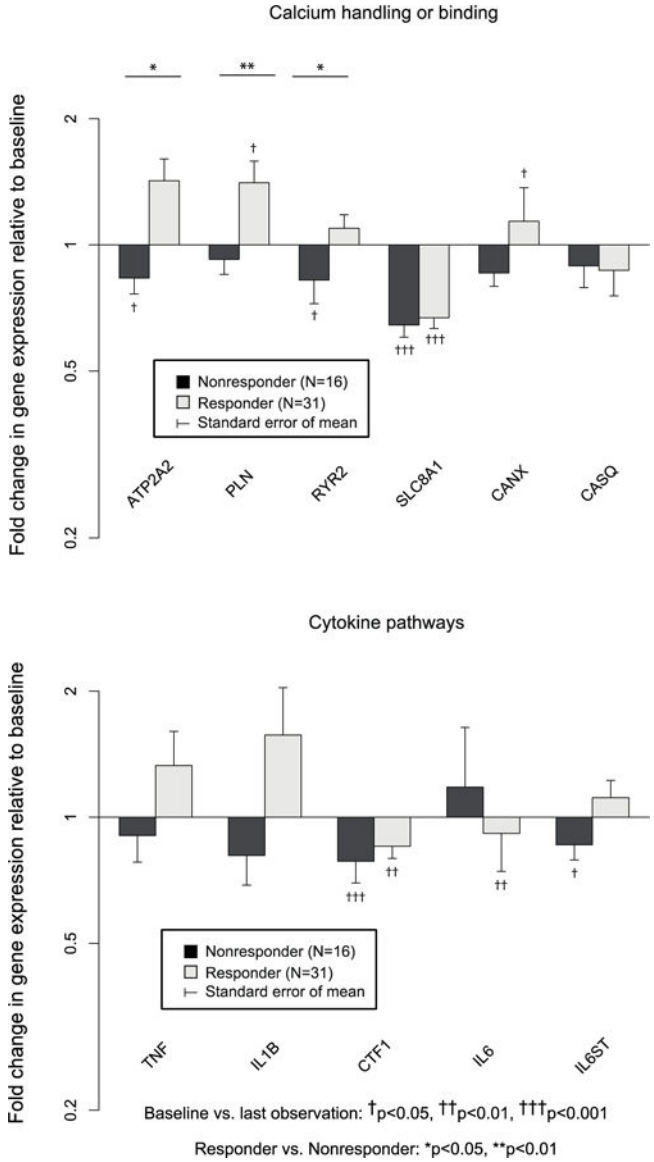

Figure 3b –. Fold change in gene expression: calcium handling and cytokine pathways in Responders (N=31) vs. Nonresponders (N=16).

ATP2A2, PLN, and *RYR2* were upregulated in Responders vs. Nonresponders.

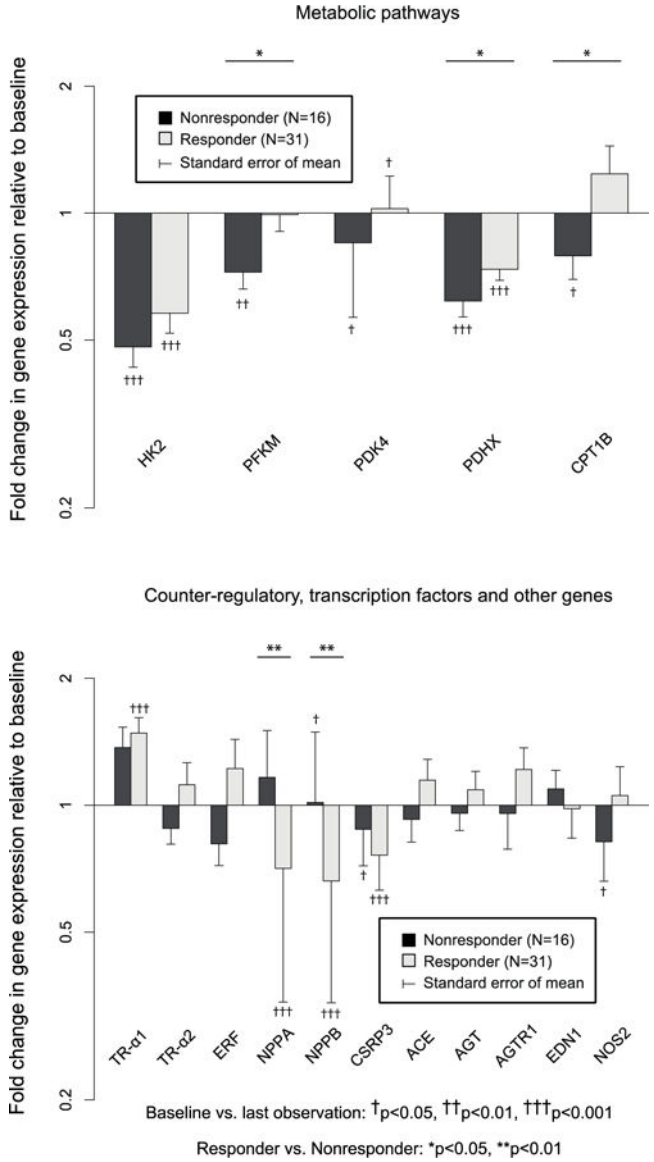

Figure 3c –. Fold change in gene expression: metabolic pathways and counter-regulatory, transcription factors, and other genes in Responders (N=31) vs. Nonresponders (N=16).

PFKM, PDHX, and *CPT1B* were upregulated in Responders vs. Nonresponders. *NPPA* and *NPPB* were downregulated in Responders vs. Nonresponders.

Table 1 –

Genes measured by RT-qPCR

Pathway	Symbol	Gene Name	Pathway	Symbol	Gene Name
Adrenergic signal transduction (N=11)	ADRB1	Adrenoceptor β1	Contractile, cytoskeleton proteins (N=12)	MYH6	Myosin, heavy chain 6, cardiac, alpha
	ADRB2	Adrenoceptor β2		MYH7	Myosin, heavy chain 7, cardiac, beta
	HNRNPD	Heterogeneous nuclear ribonucleoprotein D		ACTA1	Actin, α1, skeletal
	ADRBK1	Adrenergic, beta, receptor kinase 1		ACTC1	Actin, α1, cardiac
	GNAS	GNAS complex locus		MYL2	Myosin light chain 2, regulatory, cardiac, slow
	GNAI2	G protein, α inhibiting activity polypeptide 2		MYL3	Myosin light chain 3, alkali; vent., skeletal, slow
	ADCY5	Adenylate cyclase 5		MYL4	Myosin light chain 4, alkali; atrial, embryonic
	ADRA1A	Adrenoceptor α 1A		MYL7	Myosin light chain 7, regulatory
	GNAQ	G protein, q polypeptide		TNNT2	Troponin T type 2
	PRKCB	Protein kinase C, β		TNNI3	Troponin I type 3
	SLC9A1	Solute carrier family 9, (Na+/H + exchanger), member 1		TNNC1	Troponin C type 1
RAAS/endothelin (N=4)	ACE	Angiotensin I converting enzyme 1		DMD	Dystrophin
	AGT	Angiotensinogen		NPPA	Natriuretic peptide A
	AGTR1	Angiotensin II receptor, type 1		NPPB	Natriuretic peptide B
	EDN1	Endothelin 1	Counter-regulatory, transcription, other factors (N=6)	TR-α1	Thyroid hormone receptor-α, splice variant 1
	HK2	Hexokinase 2		TR-α2	Thyroid hormone receptor-α, splice variant 2
	PFKM	Phosphofructokinase, muscle		ERF	Ets2 repressor factor
Metabolic pathways (N=5)	PDK4	Pyruvate dehydrogenase kinase, isozyme 4		CSRP3	Cysteine and glycine-rich protein 3
	PDHX	Pyruvate dehydrogenase complex, component X		NOS2	Nitric oxide synthase 2, inducible
	CPT1B	Carnitine palmitoyltransferase 1B		TNF	Tumor necrosis factor
	ATP2A2	ATPase, Ca++ transporting, cardiac, slow twitch 2	Cytokines (N=6)	IL1B	Interleukin 1, β
	PLN	Phospholamban		CTF1	Cardiotrophin 1
	RYR2	Ryanodine receptor 2		IL6	Interleukin 6
Calcium handling or binding (N=6)	CASQ2	Calsequestrin 2		IL6ST	Interleukin 6 signal transducer
	SLC8A1	Solute carrier fam. 8 (Na+/Ca+ + exchanger), member 1		GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
	CANX	Calnexin	Normalization (N=2)	RN18S1	RNA, 18S ribosomal 1

RAAS=Renin-angiotensin-aldosterone system

Table 2a –

Baseline clinical characteristics

	IDC (all BB)	NF (Control)	F vs. NF	R	NR	R vs. NR
Clinical characteristic	**47**	**4**	**p**	**31 (66.0%)**	**16 (34.0%)**	**p**
Age, years	45.8±13.1	41.0±17.1	0.62	43.8±13.2	49.7±12.3	0.20
HF duration, months	23.5±45.5	-	-	6.8±9.8	55.8±67.0	0.001
Female	13 (27.6%)	1 (25.0%)	1	9 (29.0%)	4 (25.0%)	1
Race			1			0.58
White	31 (66.0%)	4 (100.0%)		22 (71.0%)	9 (56.2%)	
Black	6 (12/8%)	0 (0.0%)		4 (12.9%)	2 (12.5%)	
Hispanic	7 (14.9%)	0 (0.0%)		4 (12.9%)	3 (18.8%)	
Other	3 (6.4%)	0 (0.0%)		1 (3.2%)	2 (12.5%)	
NYHA class			0.002			0.55
I	0 (0.0%)	2 (50.0%)		0 (0.0%)	0 (0.0%)	
II	26 (55.3%)	2 (50.0%)		16 (51.6%)	10 (62.5%)	
III	21 (44.7%)	0 (0.0%)		15 (48.4%)	6 (37.5%)	
Atrial fibrillation	10 (21.3%)	1 (25.0%)	1	4 (12.9%)	6 (37.5%)	0.07
Hypertension	18 (38.3%)	2 (50.0%)	0.64	15 (48.4%)	3 (18.8%)	0.06
Creatinine clearance, ml/min	80.1±21.5	87.8±7.9	0.59	85.4±18.4	71.0±26.2	0.04

IDC=Idiopathic dilated cardiomyopathy; BB=β-blocker; F=Failing; NF=Non-failing; R=Responder; NR=Nonresponder

Table 2b –

Baseline cardiac parameters

	IDC (All BB)	NF (Control)	F vs. NF	R	NR	R vs. NR
Clinical characteristic	**47**	**4**	**p**	**31 (66.0%)**	**16 (34.0%)**	**p**
LVEF, %	26.2±8.9	58.8±7.4	0.001	25.6±8.2	27.3±10.5	0.68
RVEF, %	27.1±8.9	38.3±4.7	0.03	27.0±8.7	27.2±9.7	0.95
LV end diastolic vol., ml	229±92	-	-	220±83	247±110	0.50
Heart rate, bpm	84.5±20.3	74.0±18.2	0.64	86.7±21.3	80.3±17.3	0.24
QRS, ms	115±31	101±9	0.51	107±24	133±39	0.03
Norepinephrine, pg/ml	489±327	372±152	0.69	450±261	574±438	0.74
Systolic blood press., mm Hg	107±14	118±29	0.56	106±13	109±17	0.46
Mean PA pressure, mm Hg	24.1±10.5	20.0±3.0	0.59	22.6±9.8	27.1±11.5	0.18
Cardiac index, L/min/m^2	2.2±0.6	3.0±0.5	0.05	2.3±0.7	2.2±0.6	0.91

IDC=Idiopathic dilated cardiomyopathy; BB=β-blocker; F=Failing; NF=Nonfailing; R=Responder; NR=Nonresponder; PA=Pulmonary artery

Table 3 –

Changes in cardiac structure and function according to responder status.

Variable	Responder 31 (66.0%)	Nonresponder 16 (34.0%)	Responder vs. Nonresponder
LVEF, %	21.2±9.8‡	1.4±4.9	<0.0001
RVEF, %	9.8±11.4†	4.9±10.4	0.27
LV end diastolic vol., ml	−82.1±59.6‡	15.5±57.7	<0.001
Heart rate, bpm	−18.2±20.6†	−4.7±13.3	<0.001
Norepinephrine, pg/ml	−88.2±349.1	−72.1±557.7	0.69
Systolic blood pressure, mm Hg	8.0±17.4*	0.9±13.8	0.24
Mean PA pressure, mm Hg	−4.6±8.4*	1.7±8.8	<0.05
Cardiac index (L/min/m^2)	0.4±0.9*	0.1±0.7	0.17

Compared to baseline:

*p<0.05

†p<0.001

‡p<0.0001

PA=pulmonary artery

Table 4 –

Significant differential changes in gene expression in Responders vs. Nonresponders compared with known gene regulatory pathways

		Concordance with regulatory pathways			Verification	
Gene name	**Δ, R vs. NR**	***Adult/ Fetal**	**†Inverse β_1-AR stimulation**	**‡THR**	**GAPDH+18S**	**Affymetrix**
ADRB1	↑	+	+	+	✓	NS
ADRB2	↑	?	+	+	✓	✓
ADRA1A	↑	+	+	+	✓	NS
MYH6	↑	+	+	+	✓	✓
MYL3	↑	+	+	X	✓	✓
NPPA	↓	+	+	+	✓	✓
NPPB	↓	+	+	+	✓	✓
ATP2A2	↑	+	+	+	✓	NS
PLN	↑	+	+	−	✓	✓
RYR2	↑	+	+	+	✓	✓
CPT1B	↑	+	+	+	✓	NS
PDHX	↑	?	?	?	✓	NS
PFKM	↑	+	+	+	✓	✓
MYH6/MYH7	↑	+	+	+	✓	✓
ACTC1/ACTA1	↑	+	+	+	NS	✓

‡Changes in gene expression concordant with thyroid hormone stimulation or TR-α_1 expression.[7]

†Directionally opposite regulation from that produced by β_1-AR stimulation[5,36] or transgenic cardiac overexpression;[6]

*+=Concordant with pathway; −=Discordant with pathway; ?=Unknown if regulated by pathway; X=not regulated by pathway; ✓=Concordant, significant; NS=Concordant, not significant ; Δ=Directional change; R=Responder; NR=Nonresponder; THR=Thyroid hormone receptor. Decreased expression of genes expressed at higher levels in embryonic development (“fetal” genes) or increased expression of genes expressed at higher levels in the adult than in embryonic development (“adult” genes);