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Cardiac resynchronization therapy reduces expression of inflammation-promoting genes related to interleukin-1β in heart failure

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Aims In light of recent data regarding inflammatory signalling pathways in cardiovascular disease and the recently demonstrated impact of pharmacologic inhibition of interleukin-1β (IL-1β) in heart failure, the primary aim was to assess the physiologic effects of cardiac resynchronization therapy (CRT) on the expression of systemic inflammatory, immune-modulatory, metabolic, and apoptotic genes in peripheral blood mononuclear cells (PBMCs) of patients with heart failure.

Methods and results We used RNA sequencing (RNA-Seq) and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) to identify gene expression changes in PBMCs in response to CRT. In total, 27 patients were analysed: 12 with heart failure undergoing CRT, 6 with heart failure undergoing standard implanted cardioverter defibrillators, and 9 with coronary artery disease but not heart failure. In CRT patients (median age 65.5 years, interquartile range 63.0–66.8 years, 33% female), RNA-Seq analysis identified 40 genes, including multiple genes associated with the IL-1β pathway, with significant correlations (false discovery rate < 0.05) with four key CRT response measures. CRT was associated with suppression of PBMC expression of IL-1β (1.80-fold decrease, $P = 0.047$), FOS proto-oncogene (FOS) (3.25-fold decrease, $P = 0.01$), dual specificity phosphatase 1 (DUSP1) (2.05-fold decrease, $P = 0.001$), and early growth response 1 (EGR1) (7.38-fold decrease, $P = 0.03$), and suppression was greater in responders vs. non-responders ($P = 0.03$ for IL-1β, $P = 0.02$ for FOS, $P = 0.02$ for DUSP1, and $P = 0.11$ for EGR1). Baseline FOS and DUSP-1 levels were greater in responders vs. non-responders (6.15-fold higher, FOS, $P = 0.002$; 2.60-fold higher, DUSP1, $P = 0.0001$). CRT responders but not non-responders showed higher baseline gene expression of FOS ($P = 0.04$) and DUSP1 ($P = 0.06$) compared with control patients without heart failure. Baseline serum high-sensitivity C-reactive protein levels were 3.47-fold higher in CRT responders vs. non-responders ($P = 0.008$).

Conclusion Treatment of heart failure with CRT resulted in decreased PBMC expression of genes linked to inflammation. Moreover, CRT responders had higher expression of these inflammatory genes prior to CRT and greater suppression of these genes after CRT compared with non-responders.

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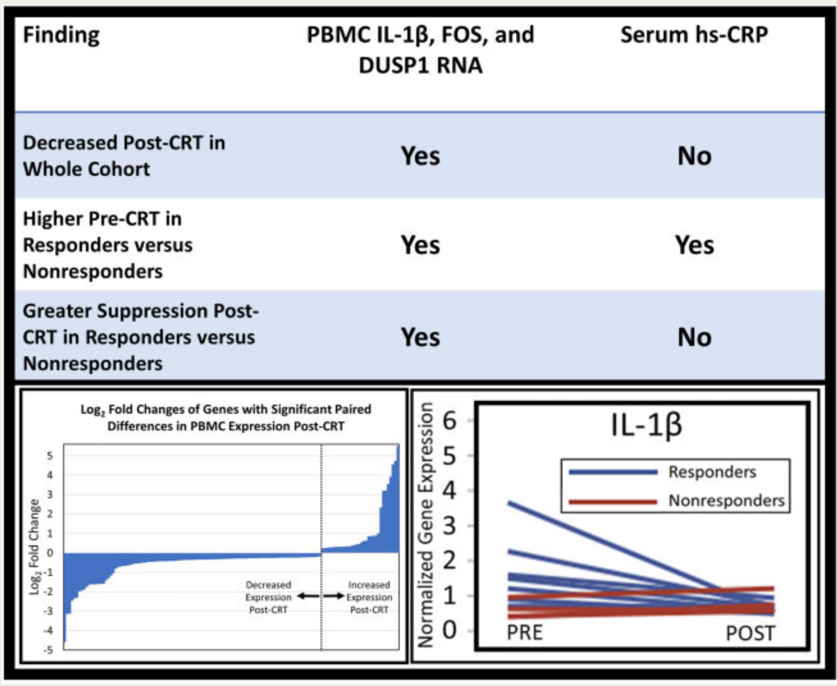
Graphical Abstract



Finding	PBMC IL-1β, FOS, and DUSP1 RNA	Serum hs-CRP
Decreased Post-CRT in Whole Cohort	Yes	No
Higher Pre-CRT in Responders versus Nonresponders	Yes	Yes
Greater Suppression Post-CRT in Responders versus Nonresponders	Yes	No

Keywords Cardiac resynchronization therapy • RNA-seq • PCR • Heart failure • Il-1β

1. Introduction

While cardiac resynchronization therapy (CRT) has been shown to be an effective treatment for many patients with heart failure,[1,2] response rates are in the range of 50–60%.[3–8] Heart failure is a progressive disease, and straightforward methods such as blood tests to predict and monitor the durability of response after therapies such as CRT could greatly improve clinical care. More generally, there is an unmet need for biomarkers that can assess response, determine prognosis, and inform the need for other therapies. Inflammation and dysregulation of the immune response are key inter-related factors involved in heart failure progression, independent of the initial form of cardiac injury.[9,10] Both innate and adaptive immune cells such as monocytes, T cells, and B cells regulate cellular processes in heart failure and display functional heterogeneity in a subset-dependent manner.[11–13] Complex interactions between different immune cell subsets and inflammatory cytokines post-myocardial injury promote cardiac repair, eccentric cardiac remodelling, and ventricular dysfunction.[9,11] In addition to the local immune-inflammatory activation, heart failure patients display increased levels of systemic inflammation, and peripheral immune cells with altered activation and inflammatory states are associated with heart failure severity and adverse clinical outcomes.[10,13,14] Recently, a sub-analysis of the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial demonstrated that reducing inflammation by targeted inhibition of interleukin-1β (IL-1β) with a monoclonal antibody (Canakinumab) reduces hospitalization for heart failure and heart failure-associated mortality in a subset of patients with prior myocardial infarction and elevated levels of the inflammatory C-reactive protein (CRP).[15] Furthermore, IL-1β blockade by both canakinumab and the IL-1 receptor antagonist, anakinra has been shown to improve peak oxygen consumption (VO_2) and left ventricular ejection fraction in subjects with systolic heart failure.[16,17]

Studies have revealed that injured myocardium sends signals to other organs triggering leucocyte proliferation with increased numbers in the circulation.[18,19] Circulating immune cells serve as messengers linking local and systemic inflammation[18] and potentially mirror local pathophysiologic processes as evidenced by transcript signatures of peripheral blood mononuclear cells (PBMCs) in heart failure patients.[20–23] Because CRT modulates the inflammatory response[24,25] in heart failure, we hypothesized that CRT also modulates expression of key genes in circulating immune cells that regulate inflammation. Identification of these genes and gene pathways in circulating PBMCs has the potential to provide unique mechanistic insights into intracellular pathways modulated by CRT and identify novel biomarkers for safe and targeted immune therapies for heart failure. Additionally, such a gene expression profile could aid physicians in tailoring recommendations for patients with heart failure with or without CRT. The present investigation aims to assess variations in the transcriptomic profile of PBMCs before and 6 months after CRT using ribonucleic acid (RNA) sequencing (RNA-Seq) microarrays and validation of differentially expressed transcripts with quantitative real-time polymerase chain reaction (RT-qPCR). In addition, we also evaluated the associations of these peripheral RNA expression changes with standard CRT response measures such as improvement in left ventricular systolic function and serum levels of high-sensitivity CRP (hs-CRP).

2. Methods

2.1 Study cohort

Twelve heart failure patients with either ischaemic or non-ischaemic cardiomyopathy with a standard guideline-based indication for CRT and six heart failure patients with an indication for the implanted cardioverter defibrillator (ICD) were recruited from the University of Virginia Health System. Nine age- and sex-matched subjects from the Coronary Assessment in Virginia Cohort (CAVA)[26] with ejection fraction >50 were used as control subjects with no heart failure. Informed consent was obtained from all participants, and the study protocol was approved by the Institutional Review Board for Human Subjects Research at the University of Virginia. This research study was performed in accordance with the principles of the Declaration of Helsinki.

2.2 Clinical data and determination of clinical response to CRT

The CRT and ICD procedures were performed using standard clinical methods. All patients had a comprehensive assessment with four clinical endpoints based on echocardiography, cardiopulmonary exercise tests, heart failure symptom scores (Minnesota Living with Heart Failure Questionnaire), and serum B-type natriuretic peptide (BNP) levels before and 6 months after CRT. Patients also had blood draws at these time points for RNA analysis and hs-CRP assessments. WBC counts were also determined for all patients before CRT and in all but two patients after CRT. In these two patients, the WBC counts post-CRT were imputed as the same values obtained pre-CRT. The percentage change in left ventricular end-systolic volume (LVESV) before and after CRT was calculated as: (post-CRT LVESV - pre-CRT LVESV)/pre-CRT LVESV. Changes in peak VO_2 and heart failure scores post-CRT were evaluated as the net paired change per patient based on typical practice. BNP changes were reported as the fractional paired change per patient. Four-year survival free of cardiac transplantation and left ventricular assist device after CRT was assessed in all patients.

2.3 Total PBMC RNA preparation

Whole blood was drawn into citrated Cell Preparation Tubes (BD Biosciences # 362761, San Jose, CA, USA) and processed within 1 h of collection. PBMCs were separated from granulocytes and red blood cells by ficoll density-gradient centrifugation, and total PBMC RNA was purified using Qiagen RNeasy Plus kit, including a DNase digest following the manufacturer's instructions (Qiagen, Valencia, CA, USA). RNA was quantified spectrophotometrically (Nanodrop, Thermo Scientific, Wilmington, DE, USA) and assessed for quality by capillary gel electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Inc., Palo Alto, CA, USA). cDNA libraries were prepared using the Illumina TruSeq RNA Sample Preparation Kit following the manufacturer's recommended procedures. Of the 12 heart failure patients, a cDNA library could not be generated successfully for one patient, resulting in RNA-Seq analysis in 11 patients, although RT-qPCR was subsequently performed from samples for all 12 patients. Libraries were sequenced using 100 paired-end reads on Illumina HiSeq 2500 instruments. Library construction and RNA-Seq were performed at the Hudson Alpha Institute for Biotechnology (Huntsville, AL, USA).

2.4 Validation of differentially expressed genes by RT-qPCR

Expression of important differentially expressed genes from RNA-Seq analysis was confirmed by quantitative RT-qPCR using Taqman probes from Applied Biosystems (Foster City, CA, USA). cDNA samples were reverse transcribed from RNA of all 12 heart failure patients using 15 ng of the cDNA per 10 μL of the Taqman RT-qPCR reaction mix (Applied Biosystems). Taqman components included 5 μL of the Taqman universal PCR master mix and 0.5 μL of the TaqMan gene expression assay probe. Results were normalized to 18S ribosomal RNA (rRNA), and relative fold changes in gene expression levels were calculated using the 2-ΔΔCt method.[27]

2.5 Data analysis and statistics

Baseline demographic and clinical characteristics of patients were expressed as medians and interquartile range (IQR) for continuous variables, while numbers and percentages were used to describe categorical variables. Bivariable logistic regression was used to evaluate differences in 4-year survival free of cardiac transplantation and left ventricular assist device in patients designated as responders or non-responders based on the change in the LVESV during the first 6 months after CRT. SAS 9.4 (Cary, NC, USA) was used for this statistical analysis.

With respect to analysis of RNA-Seq data, reads from Hudson Alpha were aligned using the Human Genome Build 38 project. A total of 22 375 transcripts were identified using Salmon and imported into R using tximport. A regularized log transformation was applied to the count data to perform quality assessment. The bioconductor package edgeR was used to identify differentially expressed genes before and after CRT using a paired design to fit a quasi-likelihood negative binomial generalized log-linear model to count data. We evaluated fold changes for differentially expressed genes before and after CRT with a Benjamini–Hochberg false discovery rate (FDR) of 0.05, which accounts for multiple comparisons. Each of the four clinical endpoints was used to perform a covariate analysis using edgeR. Changes in gene expression before and after CRT were correlated with changes in response measures before and after CRT.

Selection of genes for further analysis with RT-qPCR was based on a two-step process in which we first selected those genes with significant changes post-CRT and significant associations with CRT response, and second determined which of these genes appeared to have the greatest impact on key inflammatory pathways. In the first step, we performed an analysis of differences in gene expression for each patient before and after CRT and a correlation analysis to identify those genes most closely associated with the four clinical response measures (LVESV from echocardiography, heart failure scores, peak VO_2 from cardiopulmonary exercise testing, and serum BNP levels). Those genes ($N = 2853$) with the strongest associations with these response measures were then subjected to a pathway enrichment analysis in order to identify genes associated with a broad array of systemic inflammatory, immune-modulatory, metabolic, and apoptotic pathways. A full summary of the analytic strategy and study design are provided in *Figure 1*.

RT-qPCR gene expression levels, for which all measurements were obtained twice, were analysed using linear mixed effect models. The models included the timing of the measurement (pre-CRT vs. post-CRT), the patient's responder status (as a binary variable), the interaction between the timing of the measurement and the responder status,

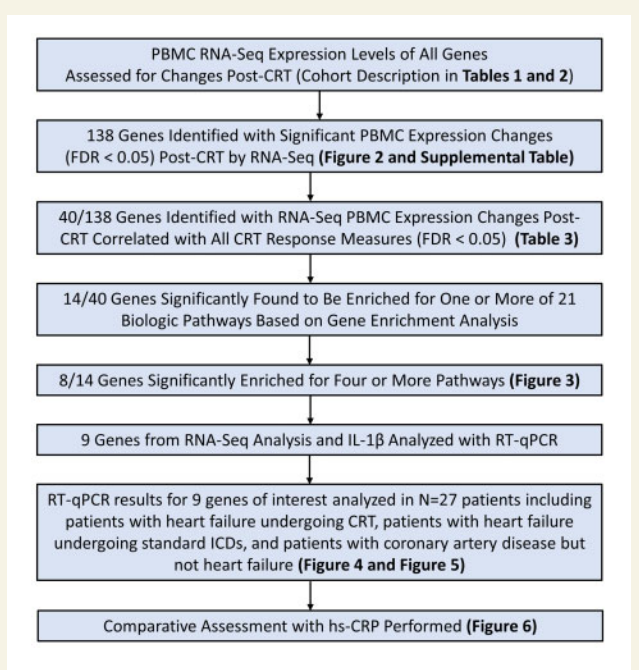


Figure 1 Summary of analytic strategy and major findings. The flow chart shows a summary of the analytic strategy for PBMC gene expression analysis using RNA-Seq and RT-qPCR for the 27 patients in the study. The locations in tables and figures of key findings are also specified. RNA-Seq, RNA sequencing; RT-qPCR, quantitative real-time polymerase chain reaction.

and replicates of each measurement. The *P*-value for the interaction term in the model was used to determine whether there was a significant difference in gene expression post-CRT in responders vs. non-responders, while the *P*-value based on the timing variable (pre-CRT vs. post-CRT) was used to evaluate whether there was a significant difference in expression post-CRT. Comparisons of baseline gene expression in two groups were also performed using *t*-tests.

3. Results

3.1 Patient characteristics

The baseline characteristics of the 27 patients in the study (12 heart failure patients undergoing CRT therapy, 6 patients with ICDs, and 9 matched controls with coronary artery disease but no heart failure) are shown in *Table 1*. The median age of CRT patients was 65.5 years (IQR 63.0–66.8 years), while the median age of control coronary artery disease (CAD) patients was similarly 65.0 years (IQR 59.0–66.0 years), and 33.3% of both groups were female.

The response parameters of the CRT patients are shown in *Table 2*. The median net improvement in peak VO_2 per patient (paired analysis) was 2.2 (IQR -0.4 to 3.6), and the median fractional improvement (decrease) in LVESV per patient (paired analysis) was -0.18 (IQR -0.30 to 0.04). Of the 12 patients, 8 had a reduction of the LVESV by at least 15% after 6 months of CRT. These eight patients were designated as CRT responders. This distinction between responders and non-responders was validated by differences in 4-year-survival free of heart transplantation and left ventricular assist device after CRT, which was 88% in responders and 25% in non-responders ($P = 0.053$).

3.2 Significant down-regulation of multiple gene transcripts after CRT

RNA-Seq was performed genome wide, and 22 375 transcripts were analysed using the Human Genome Build 38. A total of 2853 genes were analysed because they showed significant correlations with clinical endpoints based on peak VO_2, BNP levels, LVESV, and heart failure scores with an FDR of <0.05. As shown in *Figure 2* and the Supplementary material online, Supplementary *Table*, analysis of differences in gene expression for each patient showed that 138 of these 2853 genes had significant differences in expression before and after CRT based on an FDR <0.05. Of these 138 genes, 107 had suppressed levels of expression after CRT, while 31 had increased expression after CRT. The distribution of $\log_2$ fold changes in the PBMC expression of these genes post-CRT may be visualized in *Figure 2*.

3.3 Selection of candidate genes of interest for RT-qPCR analysis

A pairwise correlation analysis identified 40 genes with the strongest association with all four CRT response measures (reduction in the LVESV, a more favourable heart failure symptom score post-CRT, reduction in BNP, and improvement in cardiopulmonary exercise capacity post-CRT), based on an FDR of 0.05 (*Table 3*). We then evaluated the significance of these 40 genes with respect to biological pathways by performing a gene set enrichment analysis using the Molecular Signatures Database. We identified 20 pathways significantly enriched among these 40 genes, and 14 genes were most significantly involved in these pathways, many of which were related to inflammation, cytokine signalling, immune cell functions, metabolism, apoptosis, and stress response associated with CRT treatment. There were nine genes associated with more than one of these pathways, and eight genes were associated with four or more pathways. These eight genes and the 20 associated pathways are shown in *Figure 3*.

3.4 Confirmed down-regulation of inflammatory genes by RT-qPCR post-CRT

PBMC expression changes of the nine important genes including IL-1β (which has a significant role in the pathophysiology of heart failure[15]) and the genes identified by the pathway analysis [FOS proto-oncogene (FOS), JUNB proto-oncogene (JUNB), suppressor of cytokine signalling 3 (SOCS3), dual specificity phosphatase 1 (DUSP1), interleukin 1 receptor associated kinase 3 (IRAK3), early growth response 1 (EGR1), BCL2-like 1 (BCL2L1), and MAP-kinase activated protein kinase 3 (MAPKAPK3)] were evaluated using RT-qPCR, as these genes code for either transcription factor subunits or signalling regulatory proteins that control production of various inflammatory mediators or cellular apoptotic pathways. Moreover, based on the link of the above genes with the IL-1β signalling, we also evaluated whether CRT modulates expression of IL-1β mRNA. As shown in *Figure 4*, RT-qPCR confirmed post-CRT suppression of IL-1β (1.80-fold decrease, $P = 0.047$), FOS (3.25-fold decrease, $P = 0.01$), DUSP1 (2.05-fold decrease, $P = 0.001$ and EGR1 (7.38-fold decrease, $P = 0.03$) with a trend noted for SOCS3 (1.83-fold decrease, $P = 0.14$. The 1.92-fold decrease in JUNB was driven by a large decrease in one patient and was not statistically significant.

Table 1 Baseline characteristics

	CRT all (*N* = 12)	CRT R (*N* = 8)	CRT NR (*N* = 4)	ICD (non-CRT) (*N* = 6)	CAD (*N* = 9)
Demographics					
Age (years)	65.5 (63.0–66.8)	66.1 (55.6–67.5)	65.0 (64.2–66.0)	63.0 (56.0–65.0)	65.0 (59.0–66.0)
Gender (female)	4 (33.3)	4 (50)	0 (0)	1 (16.7)	3 (33.3)
Vital signs					
Body mass index (kg/m^2)	27.2 (24.3–31.0)	29.7 (24.3–33.8)	27.1 (23.8–27.2)	26.5 (25.0–33.0)	27.3 (27.1–28.7)
Systolic blood pressure (mmHg)	115 (108.0–129.5)	117.5 (106.0–129.5)	115.0 (111.0–126.5)	120.0 (118–121.5)	124.0 (119.0–136.0)
Diastolic blood pressure (mmHg)	64.0 (61.0–80.0)	66.0 (60.0–86.5)	64.0 (62.0–71.0)	71.0 (70.0–75.0)	63 (60–70)
Comorbid disease					
Ischaemic cardiomyopathy	6 (58.3)	4 (50.0)	2 (50.0)	3 (50.0)	0 (0)
Diabetes mellitus	6 (50.0)	4 (50.0)	2 (50.0)	3 (50.0)	2 (22.2)
Chronic kidney disease	3 (25.0)	1 (12.5)	2 (50.0)	2 (33.3)	0 (0)
Hypertension	6 (50.0)	5 (62.5)	1 (25.0)	1 (16.7)	7 (77.8)
CMR findings					
LVEF (%)	17.0 (13.5–27.0)	21.0 (10.5–27.0)	16.0 (15.5–22.0)	–	–
LVEDVI (mL/m^2)	127.6 (122.1–154.5)	127.6 (123.3–154.5)	127.9 (120.5–168.6)	–	–
LVESVI (mL/m^2)	99.3 (92.2–135.0)	99.3 (92.2–135.0)	98.8 (60.3–137.0)	–	–
RVEF (%)	35.5 (17.5–40.0)	37.0 (17.5–42.0)	32.0 (21.5–38.5)	–	–
RVEDVI (mL/m^2)	64.5 (50.2–79.3)	64.5 (45.3–71.0)	69.6 (54.3–104.6)	–	–
RVESVI (mL/m^2)	39.4 (28.5–57.7)	45.5 (24.4–57.7)	35.1 (32.1–72.4)	–	–
ECG and lab findings					
QRS (ms)	158.0 (143.0–178.0)	164.5 (145.0–180.0)	154.0 (141.0–169.0)	88.0 (78.5–97.0)	88.0 (86.0–84.0)
Q-LV (ms)	110.0 (80.0–130.0)	130.0 (110.0–152.0)	80.0[a] (50.0–80.0)	–	–
Sodium (mEq/L)	139.5 (137.5–143.0)	139.5 (136.5–143.0)	139.0 (138.0–141.5)	139.0 (137.0–139.0)	139.0 (138.0–140.0)
Creatinine (mg/dL)	1.1 (1.1–1.2)	1.1 (1.1–1.2)	1.1 (1.0–1.3)	1.0 (0.9–1.4)	0.8 (0.7–0.9)
GFR (mL/min/1.73 m^2)	70.7 (54.5.71.9)	67.4 (54.5–71.4)	71.4 (60.5–87.3)	84.0 (65.0–92.0)	89.0 (89.0–92.0)
Medications					
Beta-blocker	12 (100)	8 (100)	4 (100)	6 (100)	5 (55.6)
ACE inhibitor or ARB	12 (100)	8 (100)	4 (100)	5 (83.3)	3 (33.3)
Loop diuretic	11 (91.7)	7 (87.5)	4 (100)	5 (83.3)	2 (22.2)
Digoxin	3 (25)	1 (12.5)	2 (50)	0 (0)	0 (0)

Continuous variables are presented as median (interquartile range). Categorical variables are presented as frequency (percentage).
ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; CAD, coronary artery disease; CRT, cardiac resynchronization therapy; ICD, implantable cardioverter defibrillator; LVEDVI, left ventricular end-diastolic volume index; LVEF, left ventricular ejection fraction; LVESVI, left ventricular end-systolic volume index; NR, non-responder; Q-LV, QRS-to-left-ventricular-electrogram time; R, responder; RVEDVI, right ventricular end-diastolic volume index; RVEF, right ventricular ejection fraction; RVESVI, right ventricular end-systolic volume index.
[a]$P = 0.06$ vs. CRT responders.

3.5 Association of baseline inflammatory gene expression and inflammatory gene suppression with improvement in left ventricular systolic function after CRT

Although the primary hypothesis was that expression changes of key genes would be associated with CRT response, additional analysis was performed based on the observation that expression of genes with significant reductions in responders tended to be expressed at higher levels in responders at baseline relative to baseline levels in non-responders. Baseline levels in CRT responders and non-responders, as well as in patients with standard ICDs and patients with coronary artery disease but no heart failure for reference, are shown in *Figure 5*. Baseline levels of FOS were 6.15-fold higher ($P = 0.038$), and baseline levels of DUSP1 were 2.60-fold higher ($P = 0.013$) in responders vs. non-responders (*Figure 5*). Baseline levels of IL-1β also trended to be higher in responders (2.61-fold higher $P = 0.093$) when compared with baseline levels in the non-responders (*Figure 5*). Interestingly, CRT responders but not CRT non-responders also showed higher baseline gene expression of FOS ($P = 0.043$) and DUSP1 ($P = 0.01$) compared with the control subjects with coronary artery disease but no heart failure, indicating an altered baseline inflammatory status in the responders. Moreover, baseline expression of FOS and DUSP1 were higher or trending higher in responders vs. patients with coronary artery disease and no heart failure (FOS $P = 0.04$; DUSP1 $P = 0.06$) (*Figure 5*). In addition, FOS ($P = 0.15$) and DUSP1 ($P = 0.126$) also trended to be higher in responders when compared with the heart failure subjects with an indication for a standard (non-CRT) ICD (*Figure 5*). As shown also in *Figure 4*, in which blue lines represent changes in responders and red lines represent changes in non-responders, the interactions between pre-CRT vs. post-CRT gene expression levels and responder status in linear mixed effect models were also statistically significant for IL-1β ($P = 0.03$), FOS ($P = 0.02$), and DUSP1 ($P = 0.02$), indicating greater suppression of PBMC expression of these genes post-CRT in responders vs. non-responders. A trend for

Table 2 Response characteristics (CRT patients, $N = 12$)

B-type natriuretic peptide	
Baseline (pg/mL)	673 (295–1145)
6-Month (pg/mL)	116 (81–931)
Paired fractional change	0.63 (0.37–0.80)
Peak VO_2	
Baseline (mL/kg/min)	14 (11–15)
6-Month (mL/kg/min)	15 (13–18)
Paired net change	2.2 (-0.4 to 3.6)
Heart failure score	
Baseline	44 (23–70)
6-Month	22 (9–38)
Paired net decrease	23 (10–34)
Left ventricular end-systolic volume by echocardiography	
Baseline (mL)	152 (124–195)
6-Month (mL)	139 (93–156)
Paired fractional change	-0.18 (-0.30 to 0.04)
Survival free of heart transplantation and left ventricular assist device	
1-Year	12 (100)
4-Year	8 (67)

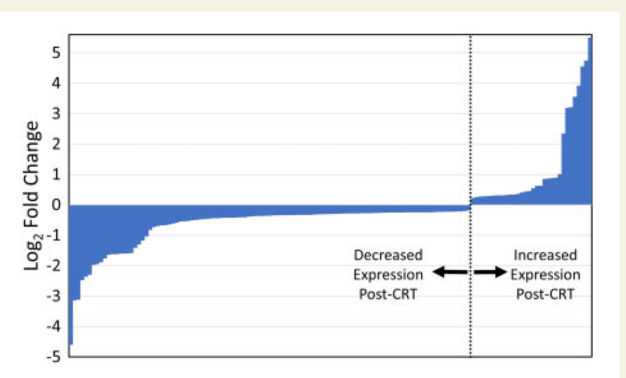


Figure 2 Log_2 fold changes of genes with significant paired differences in PBMC expression post-CRT. This graphic displays the log_2 fold changes for the 138 genes with significant (FDR < 0.05) paired differences in PBMC expression post-CRT relative to pre-CRT expression based on RNA-Seq in the 12 patients with CRT. The actual FDRs and a full list of the genes are shown in the Supplementary material online, Supplementary *Table*. CRT, cardiac resynchronization therapy; FDR, false discovery rate; RNA-Seq, RNA sequencing.

suppression was noted for IRAK3 ($P = 0.10$) and EGR1 ($P = 0.11$). With respect to aetiology of cardiomyopathy, we found no statistically significant difference in gene expression based on ischaemic aetiology of cardiomyopathy.

3.6 Comparison with serum hs-CRP levels and WBC counts before and after CRT

For comparison, serum levels of hs-CRP were assessed before and after CRT. The pre-CRT hs-CRP levels were 3.47-fold higher in responders vs. non-responders ($P = 0.008$) (*Figure 6*). Pre-CRT hs-CRP was not significantly correlated with the pre-CRT PBMC expression of IL-1β, FOS, or DUSP1. Unlike IL-1β, FOS, or DUSP1, whose expression was significantly suppressed after CRT, serum hs-CRP levels post-CRT were not significantly different from baseline levels after CRT ($P = 0.58$). Also shown in *Figure 6*, WBC counts were not significantly different pre-CRT and post-CRT in the overall cohort, and no significant differences were observed in responders and non-responders.

4. Discussion

4.1 Summary of findings

The main findings of this investigation are: (i) 138 genes, many related to inflammation, immune regulation, apoptosis, metabolism, and stress response showed significant differential expression in PBMCs post-CRT based on the RNA-Seq analysis; (ii) most of the differentially expressed genes were down-regulated after CRT; (iii) 40 of these genes were significantly correlated with all four CRT response measures, and eight in particular were noted to have prominent correlations with multiple inflammatory and other biologic pathways of interest; (iv) RT-qPCR confirmed CRT-associated suppression of many inflammatory transcripts, particularly those involved in IL-1β signalling (IL-1β, FOS, and DUSP1) and EGR1, a transcription factor that plays critical roles in various cardiovascular pathologies; (v) CRT responders had greater pre-CRT PBMC expression of these genes and greater suppression of PBMC expression of these genes post-CRT; and (vi) serum hs-CRP levels prior to CRT were higher in CRT responders compared with CRT non-responders; however, hs-CRP levels were not down-regulated post-CRT.

4.2 Associations with IL-1β and cardiovascular disease

IL-1β, an apical proinflammatory cytokine, is up-regulated in heart failure and associated with the degree of disease severity and unfavourable outcomes independent of the heart failure aetiology.[28] Multiple preclinical studies and small clinical trials have indicated the potential benefit of IL-1β inhibition with anakinra in heart failure.[29] Moreover, the recent CANTOS trial demonstrated that reducing inflammation by targeted inhibition of IL-1β reduces recurrent cardiovascular events and heart failure-associated mortality in patients with prior myocardial infarction and elevated levels of CRP.[30,31] CRT has been shown to reduce inflammation associated with heart failure,[24,25] and our data showing significant reduction of IL-1β mRNA in PBMCs with CRT further supports these findings. Of course, confirmation of changes at the protein level would strengthen this finding, and this is being evaluated through ongoing studies in our group. Of note, there are similarities and differences with respect to the effects of CRT on inflammation in heart failure and the effect of targeted pharmacologic inhibition of IL-1β in heart failure. First, while lower on-treatment hs-CRP levels were associated with better outcomes in the CANTOS trial, our findings with respect to CRT in heart failure indicate that greater suppression of IL-1β and associated inflammatory transcripts in PBMCs was strongly associated with response, although suppression of serum hs-CRP was not significantly different in CRT responders and non-responders. Even so, it is remarkable that baseline hs-CRP was higher in CRT responders vs. CRT non-responders. Second, whereas pharmacologic suppression of IL-1β in the CANTOS trial was associated with leucopenia in some patients, CRT-associated

Table 3 RNA-Seq findings for 40 genes most strongly associated with CRT response

Gene ID	Gene name	FDR for post-CRT expression	FDR for BNP correlation post-CRT	FDR for echo LVESV correlation post-CRT	FDR for HF score correlation post-CRT	FDR for peak VO_2 correlation post-CRT
ENSG00000213934	HBG1	1.64E-04	6.70E-08	1.57E-11	1.05E-04	2.29E-07
ENSG00000214921	NA	9.11E-06	1.24E-13	2.01E-12	1.23E-03	5.74E-09
ENSG00000254673	AC110275.1	3.63E-02	2.97E-09	3.50E-06	6.11E-06	4.21E-03
ENSG00000166947	EPB42	4.75E-04	1.46E-08	2.78E-11	3.85E-07	1.45E-02
ENSG00000004939	SLC4A1	7.41E-05	1.88E-22	1.88E-55	2.85E-19	3.71E-08
ENSG00000143416	SELENBP1	3.23E-05	2.26E-15	7.05E-12	5.96E-06	1.42E-02
ENSG00000133742	CA1	4.70E-04	6.57E-19	7.82E-17	3.15E-04	2.01E-07
ENSG00000122877	EGR2	7.41E-05	1.03E-04	2.68E-14	3.04E-08	1.22E-05
ENSG00000158578	ALAS2	8.24E-04	1.34E-84	1.92E-90	2.29E-28	1.69E-17
ENSG00000169877	AHSP	3.27E-03	3.42E-09	6.35E-06	1.54E-02	2.27E-02
ENSG00000120738	EGR1	**2.87E−03**	**5.42E−39**	**3.71E−89**	**1.31E−62**	**3.76E−72**
ENSG00000223609	HBD	1.82E-02	1.48E-33	1.04E-16	4.60E-07	2.93E-13
ENSG00000170345	FOS	**2.06E−07**	**6.92E−120**	**8.27E−13**	**8.60E−17**	**1.51E−36**
ENSG00000147454	SLC25A37	3.42E-02	2.48E-97	1.06E-65	1.14E-48	2.72E-56
ENSG00000196730	DAPK1	6.02E-03	2.94E-13	3.90E-20	3.41E-05	2.37E-08
ENSG00000029534	ANK1	2.57E-02	3.32E-07	3.76E-06	1.17E-08	3.82E-07
ENSG00000120129	DUSP1	**4.85E−05**	**2.63E−70**	**2.54E−12**	**4.97E−18**	**1.24E−34**
ENSG00000162722	TRIM58	3.00E-02	4.72E-33	4.76E-16	6.54E-15	2.00E-06
ENSG00000183508	FAM46C	3.99E-02	3.07E-05	3.85E-16	2.57E-11	6.68E-15
ENSG00000096060	FKBP5	1.12E-04	1.42E-16	1.01E-36	9.47E-23	2.73E-16
ENSG00000184557	SOCS3	**6.02E−03**	**2.75E−29**	**1.40E−04**	**3.73E−08**	**3.29E−07**
ENSG00000082146	STRADB	3.58E-02	2.96E-13	1.51E-05	2.44E-07	5.41E-14
ENSG00000172331	BPGM	2.87E-02	5.31E-21	1.17E-03	1.09E-10	2.26E-09
ENSG00000171552	BCL2L1	6.58E-03	1.65E-32	4.24E-12	1.77E-18	1.39E-13
ENSG00000173334	TRIB1	6.02E-03	3.72E-02	6.27E-05	1.18E-02	3.02E-04
ENSG00000153815	CMIP	3.11E-02	1.53E-14	1.39E-09	1.33E-02	4.31E-28
ENSG00000128016	ZFP36	1.50E-03	1.06E-10	8.88E-19	3.46E-02	9.55E-08
ENSG00000165406	MARCH8	3.10E-02	1.58E-06	5.53E-14	3.17E-06	4.51E-05
ENSG00000154122	ANKH	4.83E-02	1.05E-06	4.33E-07	2.24E-04	4.15E-04
ENSG00000159388	BTG2	1.14E-02	3.01E-03	2.05E-24	1.28E-03	7.34E-11
ENSG00000198876	DCAF12	1.58E-02	2.13E-13	2.51E-04	2.56E-09	3.11E-10
ENSG00000114738	MAPKAPK3	7.03E-03	1.74E-02	1.44E-03	1.98E-02	1.90E-02
ENSG00000171223	JUNB	**3.58E−02**	**2.20E−31**	**1.84E−05**	**1.36E−05**	**1.89E−21**
ENSG00000124098	FAM210B	2.77E-02	6.63E-13	2.30E-03	3.32E-06	4.22E-05
ENSG00000165671	NSD1	4.72E-02	2.61E-05	3.41E-15	2.20E-02	9.27E-03
ENSG00000103342	GSPT1	1.80E-02	3.10E-13	3.62E-04	2.15E-09	1.25E-07
ENSG00000090376	IRAK3	**4.07E−02**	**4.66E−02**	**1.23E−02**	**1.20E−02**	**2.92E−06**
ENSG00000147416	ATP6V1B2	1.23E-02	2.00E-02	6.60E-03	1.01E-04	9.11E-03
ENSG00000213145	CRIP1	1.23E-02	2.19E-03	1.06E-07	7.36E-06	1.24E-06
ENSG00000225748	PRRC2A	3.70E-02	4.66E-08	1.03E-28	7.59E-09	2.95E-43

The genes in bold represent the top genes enriched in the pathway analysis.

reductions in PBMC expression levels of IL-1β and associated transcripts were not associated with any significant changes in WBC counts.

4.3 PBMC transcripts associated with IL-1β signalling altered by CRT

FOS and JUNB are immediate-early proteins that form the proinflammatory transcription factor AP-1. Activation of AP-1 plays an essential role in cardiac remodelling, hypertrophy, and ventricular dysfunction.[32–34] JUNB has been shown to modulate macrophage activation and expression of various proinflammatory mediators such as tumour necrosis factor-α (TNF-α), IL-1β, and IL-12,[35] higher levels of which are present in heart failure patients and associated with unfavourable outcomes.[14] Moreover, AP-1 and NF-κB activation underlie β-adrenoreceptor-mediated induction of IL-6,[36] another prominent cytokine considered a biomarker as well as a potential therapeutic target for heart failure and other cardiovascular disorders.

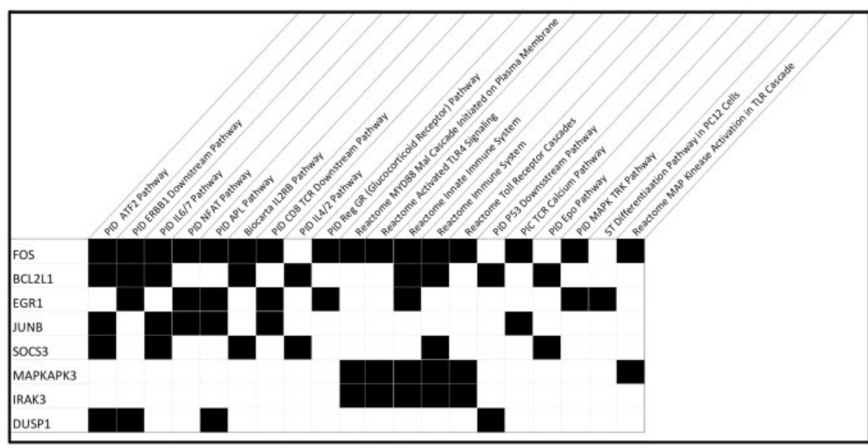


Figure 3 Pathway analysis. Of the 14 genes enriched in the pathway analysis for the 12 CRT patients, the 8 genes with involvement of 4 or more pathways are shown with the associated pathways marked. Expression of these eight genes and IL-1β were evaluated with RT-qPCR. CRT, cardiac resynchronization therapy; FDR, false discovery rate; HF, heart failure; IL-1β, interleukin-1β; LVESV, left ventricular end-systolic volume; RT-qPCR, quantitative real-time polymerase chain reaction.

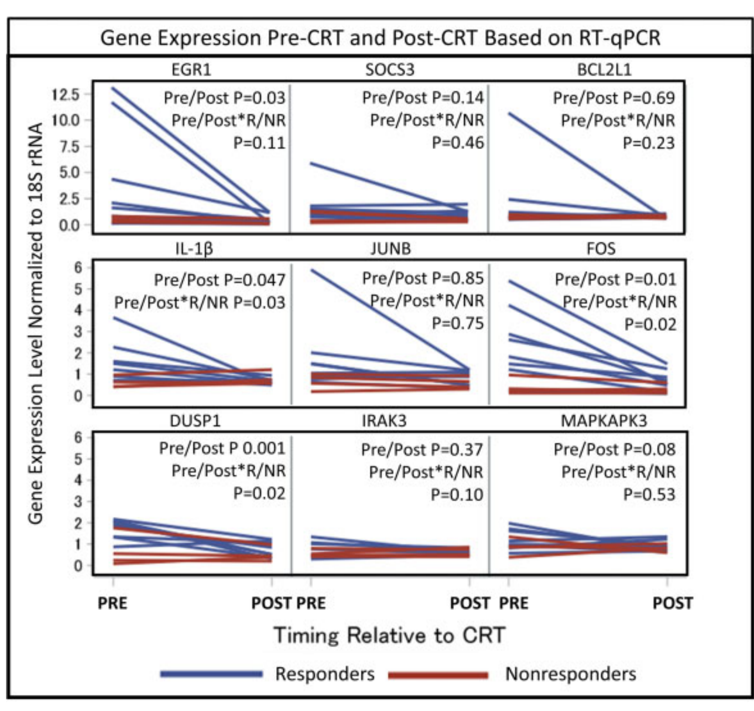


Figure 4 Gene expression pre-CRT and post-CRT based on RT-qPCR. Measured gene expression levels normalized to 18S rRNA based on RT-qPCR in the 12 CRT patients are shown for the following genes: IL-1β, FOS, DUSP1, JUNB, SOCS3, IRAK3, EGR1, BCL2L1, and MAPKAPK3. Blue lines indicate CRT responders (R), while red lines indicate non-responders (NR). *P*-values were determined using linear mixed effect models, which account for the timing of the measurement (pre-CRT vs. post-CRT), the patient's responder status (as a binary variable), the interaction between the timing of the measurement and the responder status, and replicates of each measurement. BCL2L1, BCL2-like 1; CRT, cardiac resynchronization therapy; DUSP1, dual specificity phosphatase 1; EGR1, early growth response 1; FOS, Fos proto-oncogene; IL-1β, interleukin-1β; IRAK3, interleukin 1 receptor associated kinase 3; JUNB, JunB proto-oncogene; MAPKAPK3, MAP-kinase activated protein kinase 3; RT-qPCR, quantitative real-time polymerase chain reaction; SOCS3, suppressor of cytokine signalling 3.

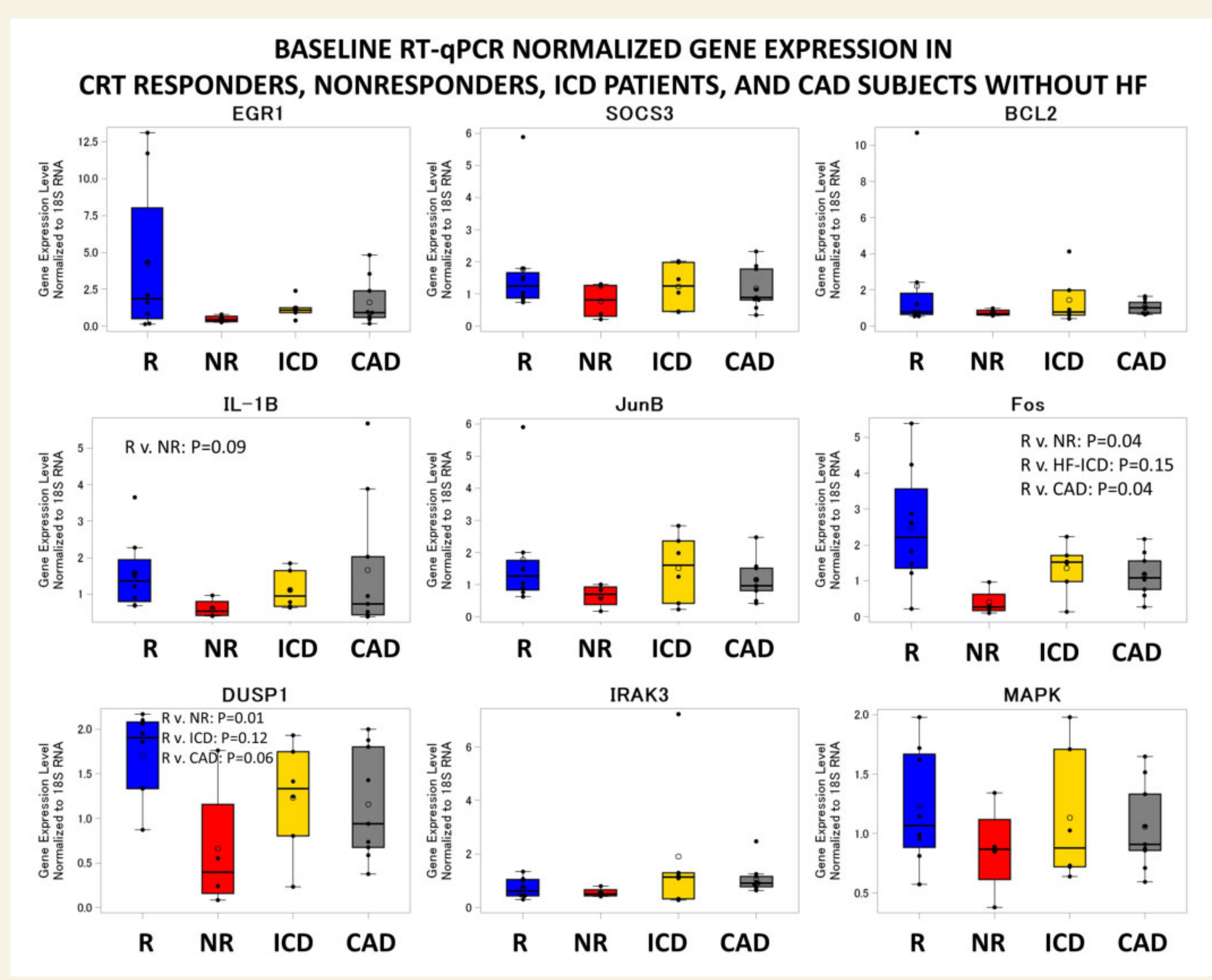


Figure 5 Gene expression changes at baseline based on RT-qPCR. Baseline levels of the 9 genes of interest based on RT-qPCR in the 27 study patients are shown in CRT responders (R), CRT non-responders (NR), patients with standard (non-CRT) ICDs, and patients with coronary artery disease but no heart failure. *P*-values reported for baseline expression differences between responders and another group were obtained using *t*-tests. BCL2L1, BCL2-like 1; CAD, coronary artery disease; CRT, cardiac resynchronization therapy; DUSP1, dual specificity phosphatase 1; EGR1, early growth response 1; FOS, Fos proto-oncogene; ICD, implantable cardioverter defibrillator; IL-1β, interleukin-1β; IRAK3, interleukin 1 receptor associated kinase 3; JUNB, JunB proto-oncogene; MAPKAPK3, MAP-kinase activated protein kinase 3; RT-qPCR, quantitative real-time polymerase chain reaction; SOCS3, suppressor of cytokine signalling 3.

DUSP1 plays a pivotal role in heart disease as a major negative modulator of the MAP kinase signalling pathways that co-ordinate diverse events including inflammation and cardiac remodelling.[37,38] DUSP1 predominantly dephosphorylates and inhibits p38 and JNK, and to a lesser extent ERK.[39] DUSP1 negatively regulates production of proinflammatory cytokines, and evidence suggests that it represses maladaptive cardiac hypertrophy.[38,40] Proinflammatory mediators, such as LPS and IL-1β, growth factors, and stress could induce transient DUSP1 expression.[39,41] Notably, DUSP1 expression was found to be up-regulated in the hearts of patients with end-stage heart failure and pressure-overloaded cardiomyopathy.[42,43] Together, these indicate that induced expression of DUSP1 under chronic inflammatory state may serve as a feedback control mechanism to limit inflammation and pathological consequences.[42]

EGR1, an immediate early gene and a zinc finger transcription factor, plays critical roles in a myriad of cardiovascular pathological processes including atherogenesis, ischaemia–reperfusion damage, cardiac hypertrophy, angiogenesis, and intimal thickening after acute vascular injury.[44] Immune cells and vascular cells such as endothelial cells, smooth muscle cells, cardiac fibroblasts, and cardiomyocytes express EGR1. Stress stimuli, proinflammatory cytokines, and growth factors are known to induce expression of EGR1, which, in turn, induces expression of a variety of proinflammatory mediators such as TNF-α and IL-6.[45] Interestingly, IL-1β has been shown to induce EGR1 expression, promoting downstream production of IL-6, IL-8, and MCP-1.[46,47] Higher expression of EGR1 transcripts as well as the EGR protein has been reported in PBMCs of chronic heart failure patients as compared with control subjects.[20] Consistent with these observations, our data also showed trending higher baseline expression of EGR1 in CRT responders who seemed to have a more inflammatory state as compared with the non-responders and control subjects with no heart failure.

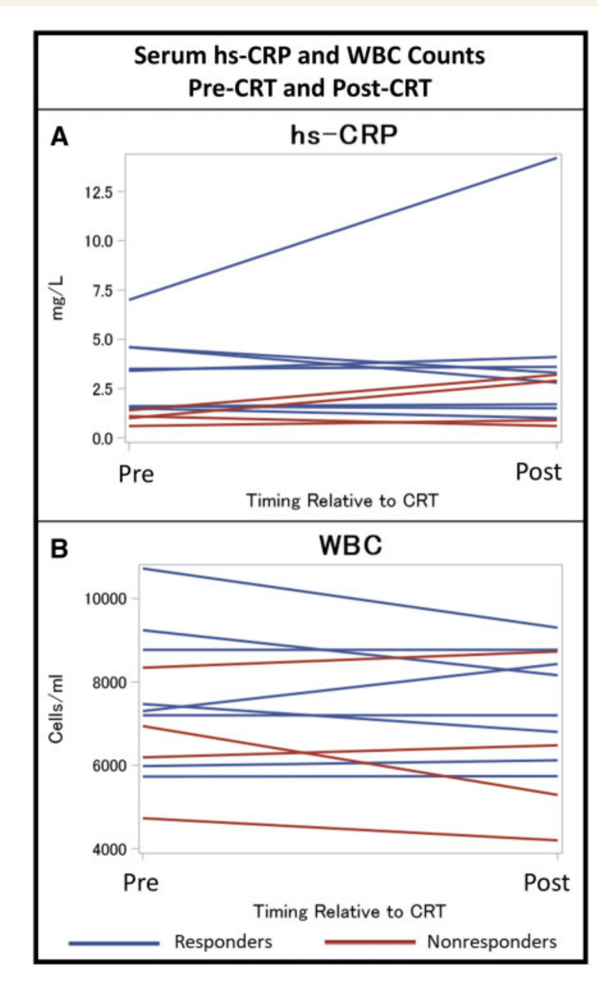


Figure 6 Serum hs-CRP and white blood cell counts pre-CRT and post-CRT. Levels of serum hs-CRP and white blood cell counts before and after CRT in the 12 CRT patients are shown for responders and non-responders. CRT, cardiac resynchronization therapy; hs-CRP, high-sensitivity C-reactive protein.

4.4 Comparison with studies of cardiac myocyte changes in response to CRT

Our approach represents a novel and complementary approach to previous studies in animals of cardiac cellular and gene expression changes in response to CRT.[48–55] This prior work has been immensely informative in demonstrating how treating abnormalities in regional contraction patterns in heart failure can alter both regional and global cellular changes in cardiac myocytes. Application of cardiac tissue analysis in humans is less feasible because it requires cardiac tissue biopsy, which is invasive and associated with some procedural risks. In contrast, PBMC analysis offers a non-invasive assessment of global physiologic changes in response to CRT that can be easily repeated at serial time points.

4.5 Implications for CRT response

The assessment of PBMC gene expression changes offers a novel insight into a different measure of CRT response. While CRT response is often considered in terms of left ventricular functional measures, short-term symptom reduction, exercise capacity, and reduction in clinical events, PBMC gene expression changes may be considered to represent another dimension of CRT response. In this present analysis, we have demonstrated that CRT is associated with a reduction in key inflammatory pathways, particularly those associated with IL-1β signalling based on both RNA-Seq and RT-qPCR analysis of PBMCs. The strongest associations with CRT response and gene expression were noted for IL-1β, FOS, DUSP1, and EGR1. Of note, identification of responders and non-responders based on left ventricular reverse remodelling was validated by the greater long-term survival free of cardiac transplantation and left ventricular assist device (88% vs. 25%) 4 years after CRT in responders vs. non-responders.

In addition to being suppressed after CRT in the entire cohort, IL-1β, FOS, DUSP1, and EGR1 had both greater suppression in responders vs. non-responders and greater baseline levels pre-CRT in responders vs. non-responders. In addition, serum hs-CRP was also higher at baseline in responders vs. non-responders; however, hs-CRP was not significantly suppressed post-CRT. As a result, assessment of CRT patients based on PBMC expression of IL-1β, FOS, DUSP1, and EGR1 has an advantage over assessment based on serum hs-CRP. In summary, these findings support the existence of an inflammatory PBMC phenotype associated with CRT response based on PBMC gene expression of inflammation-promoting genes.

4.6 Limitations

As noted earlier, we studied a relatively small number of patients in this proof-of-concept study. Even so, with high-quality RNA-Seq, RT-qPCR data, and response data, it is remarkable that significant associations confirming our hypothesis were still demonstrated in our analysis (which included adjustments for multiple comparisons based on assessment of FDRs) in a cohort of this size. Furthermore, the conclusions are supported by biologic plausibility, with a recent role demonstrated for IL-1β in other larger cohorts of patients with cardiovascular disease. These findings provide the rationale for much needed larger cohort studies of CRT patients to confirm these findings and assess associations between PBMC expression of inflammatory genes and long-term survival after CRT in all populations including minorities. Based on these findings, we expect that PBMC gene expression findings may be helpful in risk stratification after CRT and early identification of patients who may benefit from advanced heart failure therapies.

5. Conclusions

Treatment of heart failure with CRT resulted in decreased PBMC expression of genes linked to inflammation. Additionally, baseline levels of inflammatory genes prior to CRT were higher in CRT responders. Moreover, CRT responders had greater suppression of these genes after CRT compared with non-responders.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Translational perspective

Assessment of peripheral blood mononuclear cell expression of inflammatory genes before and after cardiac resynchronization therapy (CRT) may be useful for characterizing CRT response at the gene expression level and identifying patients most likely to benefit from this therapy. As a more direct measure of the biologic response to CRT compared with typical response metrics such as left ventricular volumes and symptom scores, peripheral inflammatory gene expression offers an exciting window into molecular changes occurring in response to CRT. In the future, a peripheral inflammatory gene expression profile could be developed and checked at regular intervals to monitor inflammatory physiology in heart failure patients.

Corrigendum

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This has now been corrected in the article.

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