

# Regional protein expression changes within the left ventricle in a mouse model of dyssynchronous and resynchronized heart failure

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## Abstract

**Aims** The biological mechanisms conveying the salutary effects of cardiac resynchronization therapy in heart failure remain elusive. We have recently developed a mouse model of heart failure with dyssynchrony/resynchronization. The aim of this study was to characterize regional left ventricular heterogeneity in protein expression comparing early (septum) and late (lateral) activated left ventricular wall segments in synchronous (SHF), dyssynchronous (DHF), and resynchronized heart failure (RHF).

**Methods and results** Mice subjected to ischaemia/reperfusion were divided into three groups: sinus rhythm for 4 weeks (SHF), right ventricular pacing for 4 weeks (DHF), and right ventricular pacing for 2 weeks and 2 weeks of sinus rhythm (RHF). Relative concentrations of 92 proteins from septal and lateral left ventricular wall segments ( $n = 10$  per group) were compared *within* each group. We also analysed the effect of DHF vs. SHF and RHF vs. DHF on protein expression pattern comparing the same left ventricular segments between the groups. Proteins with significantly differential expression between left ventricular segments were analysed for protein–protein correlations, protein–protein interactions, and biological and signalling pathways. Eight proteins were significantly down-regulated in the late activated (compared with early activated) lateral wall uniquely in RHF ( $P < 0.05$  adjusted for a 5% false discovery rate): *ErbB4*, *Ntf3*, *Pdgfb*, *Tnf*, *Notch3*, *Qdpr*, *Tpp1*, and *Itgb6*. Protein correlation matrix showed that six of these were strongly and positively correlated and five had known protein–protein interactions. Biological pathways mainly down-regulated in late activated myocardium in RHF were MAPK signalling and hypertrophic cardiomyopathy. There were no significantly differentially expressed proteins comparing the same left ventricular segments between the DHF and SHF (range of  $P$ -values: 0.05–1.00) and RHF and DHF (range of  $P$ -values: 0.32–1.00).

**Conclusions** In a mouse model of heart failure with dyssynchrony and resynchronization, we observed down-regulation of several proteins in the late activated lateral wall, compared with the septum, in resynchronized mice. These proteins display significant protein–protein correlation and share biological signalling pathways, including MAPK activation and hypertrophy signalling.

**Keywords** Heart failure; Cardiac resynchronization therapy; Pacing; Cell biology

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## Introduction

Cardiac resynchronization therapy (CRT) reverses remodelling and reduces morbidity and mortality in patients with heart failure and a dyssynchronous left ventricular activation pattern.<sup>1,2</sup> The biological mechanisms involved in dyssynchrony and resynchronization in failing hearts remain incompletely understood.

Left bundle branch block and right ventricular pacing result in a dyssynchronous left ventricular activation pattern where the inter-ventricular septum contracts early and the left ventricular later wall contracts late.<sup>3</sup> This dyssynchronous electrical activation pattern induces left ventricular regional differences in loading, contractile work, hypertrophy, myocardial blood flow, and oxygen consumption (all elevated in late activated left ventricular lateral wall segments).<sup>3–7</sup>

Several of these mechanisms are known to alter the transcriptome in heart failure.<sup>8,9</sup> Indeed, a previous canine study demonstrated that dyssynchrony induces regional differences in gene expression within the left ventricle (comparing early and late activated myocardium), which was nearly normalized with resynchronization.<sup>10</sup> Although useful for studying biological mechanisms, canine models are not genetically modifiable. Therefore, we have recently developed a mouse model of dyssynchrony and resynchronization and could demonstrate a dyssynchrony-induced marked heterogeneity in regional left ventricular gene expression also in that model,<sup>11</sup> but the effect of resynchronization was not evaluated.

Resynchronization-induced regional differences in left ventricular gene and protein expression may be important mediators of the beneficial effect of CRT on reverse remodelling, but the contribution of dyssynchrony and resynchronization on regional differences in protein expression remains largely unknown.

## Aims

The primary aim was to characterize left ventricular heterogeneity in protein expression in a mouse model of synchronous (SHF), dyssynchronous (DHF), and resynchronized heart failure (RHF). A secondary aim was to analyse protein–protein correlations and interactions, and molecular signalling involved based on the protein profiling.

## Methods

We have recently described a mouse pacemaker system facilitating continuous atrial and right ventricular pacing in mice for 4 weeks.<sup>11</sup> In this model, right ventricular pacing acutely induced marked electromechanical dyssynchrony that when applied continuously for 4 weeks in mice with pre-existing heart failure [ischaemia/reperfusion (I/R)] accelerated left ventricular dilatation and reduced systolic function consistent with worsening heart failure. When sinus rhythm was reinstated at 2 weeks (resynchronization), left ventricular systolic function improved, and volumes were reduced. In this study, we used cardiac tissue from mice with I/R and 4 weeks of sinus rhythm (SHF), I/R, and 4 weeks of right ventricular pacing (DHF) and I/R and 2 weeks of right ventricular pacing followed by 2 weeks of sinus rhythm (RHF). Septal (early activated) and lateral (late activated) left ventricular segments from 10 mice per group were harvested and frozen for subsequent protein quantification.

To accomplish our primary aim, we compared protein expression levels between the lateral wall and septum within each group. We also performed a between-group analysis comparing the same segments between DHF and SHF, and

between RHF and DHF. Lysate for protein quantification was produced by mixing frozen tissue samples in radioimmunoprecipitation lysis buffer using a mechanical homogenizer. The lysate was then centrifuged (8000 rpm for 10 min) before pipetted on a 96-well plate for subsequent analysis.

An exploratory mouse protein panel using enzyme-linked immunosorbent assay methodology (Olink, Uppsala, Sweden) was used to measure relative protein concentrations of 92 proteins from the two left ventricular segments (a full list of proteins analysed can be found at <https://www.olink.com/products/mouse-exploratory/biomarkers/>). This particular platform was used because it simultaneously measures relative quantification of a large number of proteins reflecting a broad range of biological processes, which suits this exploratory study. Moreover, all investigated proteins are detectable in peripheral blood in humans, increasing the translational application of the results.

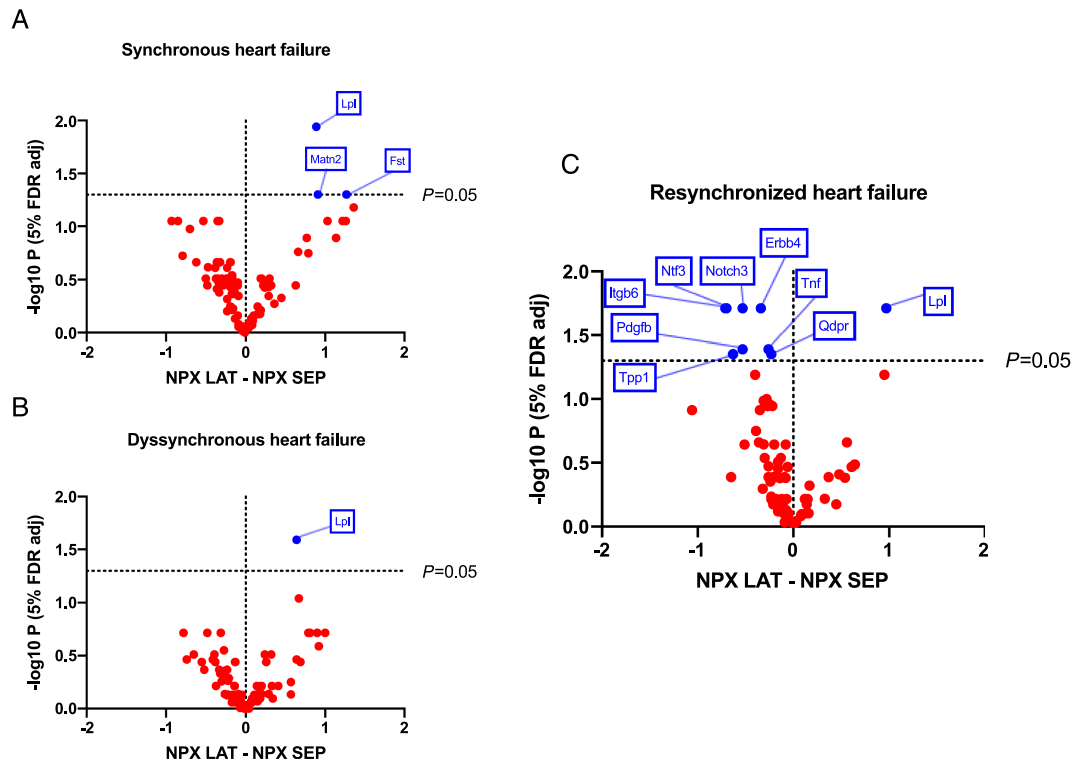
Statistical significance of within group differences in protein expression levels [expressed as normalized protein expression (NPX)] between left ventricular segments were tested with paired *t*-tests applying a 5% false discovery rate to control for multiple comparisons. Protein–protein correlations were determined by building a correlation matrix using Z-scores of the NPX difference between early and late activated segments across all samples and graphically illustrated using the *qgraph* package in R.<sup>12</sup> String database was used to predict protein–protein interactions of proteins with significantly differential expression levels within each group.<sup>13</sup> Biological and signalling pathway analyses of differentially expressed proteins were explored using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology.

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

## Results

In RHF, eight proteins were uniquely down-regulated in the late activated left lateral wall segment compared with septum (*Figure 1*). Significantly fewer proteins displayed heterogenic left ventricular expression in SHF and DHF. Correlation matrix analysis (*Figure 2A*) showed that six of the proteins differentially expressed in early and late activated myocardium in RHF were strongly and positively associated (ErbB4, Ntf3, Itgb6, Pdgfb, Tpp1, and Qdpr), while Notch 3 and TNF both displayed weaker positive correlations with the other proteins. Analysis of predicted protein–protein interactions between proteins with significant differential regional left ventricular expression in RHF using curated databases and text mining (String database) revealed a linear link between Tnf, Pdgfb, Ntf3, ErbB4, and Itgb6 (*Figure 2B*).

**FIGURE 1** (A–C) Resynchronized heart failure displays eight proteins that are uniquely down-regulated in the lateral wall segment of the left ventricle. Volcano plots displaying regional differences in protein expression [expressed as normalized protein expression (NPX)] between septum (SEP) and lateral (LAT) wall left ventricular segments. The X-axis displays differences in NPX between septum and lateral wall, and because NPX is on a log<sub>2</sub> scale, an NPX difference of 1 denotes a doubling of protein level. Y-axis displays the  $-\log_{10}$  *P*-value of difference between NPX from lateral and septal segments (*P*-values have been adjusted by applying a 5% false discovery rate).



KEGG pathway analysis defined three signalling pathways significantly down-regulated in the lateral wall in RHF: dilated cardiomyopathy, hypertrophic cardiomyopathy, and MAPK signalling pathway. The 10 most significantly affected biological pathways with cardiovascular relevance (Gene Ontology) are displayed in *Figure 2D* and included regulation of MAPK cascade, kinase activity, and cell proliferation.

The between-group analysis showed no significant differences in protein expression comparing DHF and SHF (range of *P*-values: 0.05–1.00 for septum and 0.95–0.98 for lateral wall) and RHF and DHF (range of *P*-values: 0.86–1.00 for septum and 0.32–1.00 for lateral wall).

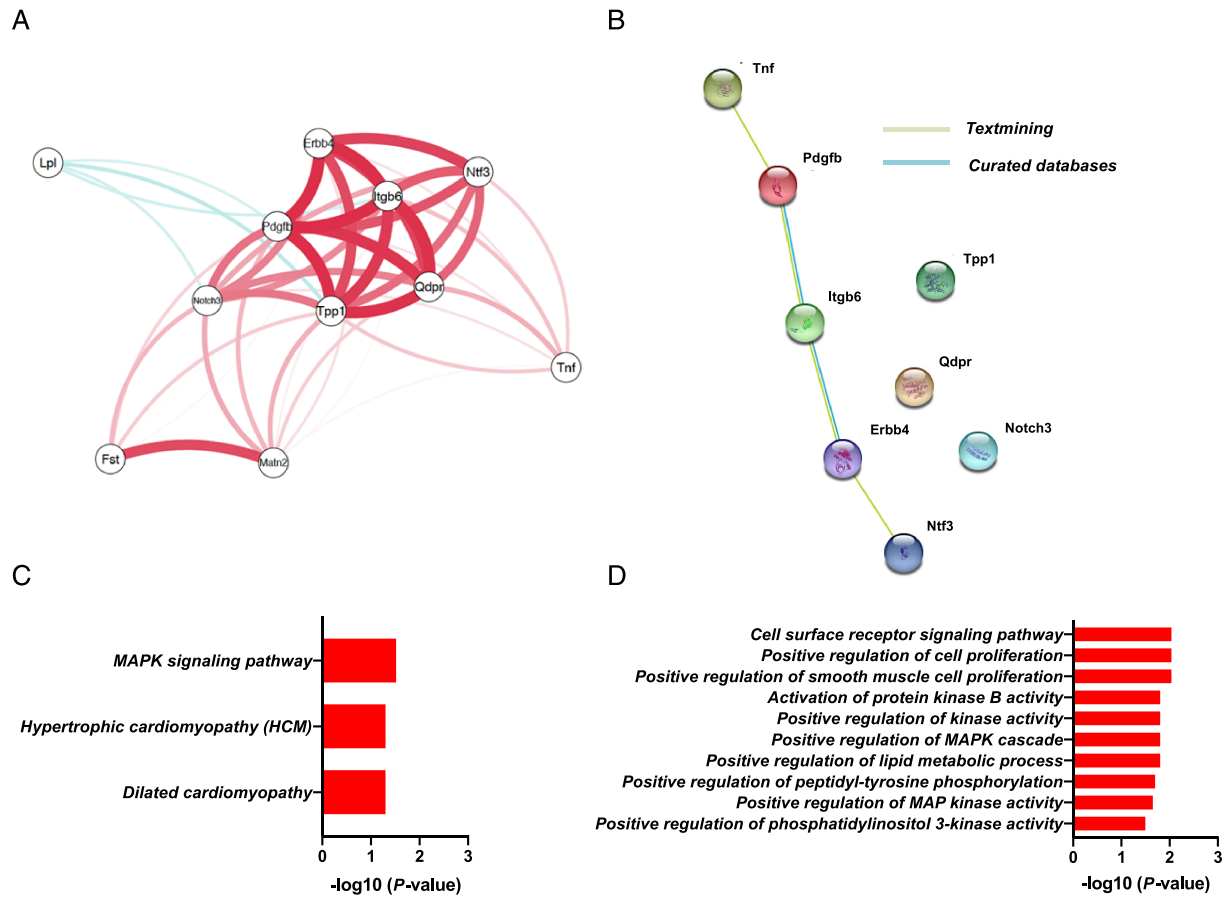
## Discussion

In this study, we used a novel mouse pacemaker model to demonstrate regional differences in protein expression within the left ventricle in resynchronized mice.

Proteins with differential expression pattern are mainly involved with MAPK, hypertrophic cardiomyopathy, and cell proliferation signalling.

Experimental studies in canines and mice using mRNA sequencing and gene array technology have previously shown that dyssynchrony induces a heterogenic gene expression pattern within the left ventricle, which is near normalized with resynchronization.<sup>10,11</sup> Hence, dyssynchrony seems to induce a regional shift in the transcriptome that is amendable with resynchronization. In our study, dyssynchrony *per se*, surprisingly, did not affect regional intra-left ventricular expression of the 92 proteins investigated, while eight proteins were down-regulated in the late activated left ventricular wall in mice subjected to resynchronization. It was also unexpected that dyssynchrony and resynchronization had no effect on protein expression when comparing the same left ventricular segments between the groups. There are some potential explanations to these findings. First, the purpose of the present study was not to recapitulate the results from mRNA sequencing/array studies (which would require mass spectrometry proteomics) but rather to describe the effect of DHF and RHF on regional protein expression patterns in an effort to discover novel proteins involved in DHF and RHF biology. The use of the 92-protein platform is suitable for our aim but is limited considering that there are tens of thousands of proteins

**FIGURE 2** Protein–protein correlation, predicted protein interactions, and signalling pathways of proteins with regional left ventricular differences in expression levels. (A) Protein–protein correlation matrix of the 11 proteins with regionally different left ventricular expression levels in all experimental groups. Red lines indicate a positive correlation, and blue lines indicate a negative correlation and a wider line indicates a stronger correlation. (B) Predicted protein–protein interaction of the eight proteins with differential expression levels between early and late activated left ventricular segments in resynchronized heart failure. The figure was extracted from String database.<sup>13</sup> (C) Significantly affected Kyoto Encyclopedia of Genes and Genomes (KEGG) signalling pathways in resynchronized heart failure. (D) Top 10 significantly affected biological pathways with cardiovascular relevance (Gene Ontology) analysing the eight proteins with significantly different expression levels between left ventricular segments in resynchronized heart failure.



in the mouse. Hence, the lack of difference in protein expression between segments in DHF and between the groups should not be interpreted as an absence of biological effect in DHF because we only investigated a small fraction of proteins in this exploratory study. Moreover, previous studies have shown that many of the important biological effects of dyssynchrony and resynchronization are mediated by post-translational modifications rather than changes in total protein expression, which were not captured in our study.<sup>14,15</sup>

Here, we report that several proteins in the MAPK signalling pathway were significantly down-regulated in the late activated lateral wall in RHF. This finding is supported by previous published data showing that MAPK also at the mRNA level is activated in the late activated myocardium in DHF in both canine (non-ischaemic) and mouse (ischaemic) heart failure models<sup>10,11</sup> and deactivated with

resynchronization.<sup>10</sup> Moreover, deactivation of MAPK with RHF has previously also been shown at the protein level (e.g. P38/JNK module, AKT, and ERK) in both ischaemic mouse and non-ischaemic canine models.<sup>11,14</sup> However, the regional disparities induced by RHF on the MAPK regulating proteins ErbB4, Ntf3, and Pdgfr have previously not been reported in RHF and constitute a novel finding.

TNF- $\alpha$  is known to be down-regulated in late activated left ventricular segments with resynchronization in a non-ischaemic canine heart failure model.<sup>14</sup> In the present study, we found down-regulation of TNF- $\alpha$  in the lateral wall (compared with the septum) in resynchronized mice but not in SHF or DHF, indicating that regional down-regulation of TNF- $\alpha$  between left ventricular segments may play a role in resynchronization. The present study also for the first time links the change in TNF- $\alpha$  to six other proteins by protein–protein correlation and

interaction analysis. The exact interplay between these proteins is outside the scope of this study, but none of these proteins have previously been associated with dyssynchrony and resynchronization and therefore adds important information to the field.

Canine studies have shown dyssynchrony-induced regional hypertrophy in late activated myocardium,<sup>6</sup> and we have previously found this to be associated with increased mRNA gene expression involved in hypertrophic signalling in the lateral wall in the mouse model.<sup>11</sup> The finding in study extends this knowledge by presenting regional changes in hypertrophy signalling at the protein level of Itgb6 and TNF.

A limitation of this study is that we did not perform confirmatory western blot experiments, mainly owing to limited amount of available tissue. Therefore, expression differences of individual proteins should be interpreted with caution. However, the findings that several proteins are involved in specific biological processes and that significant protein–protein correlations exist strengthen the validity of the results.

## Conclusions

In a mouse model of ischaemic cardiomyopathy, we found down-regulation of several proteins in the late activated left ventricular lateral wall compared with the septum in resynchronized mice.

These proteins display significant protein–protein correlation and share biological signalling pathways, including MAPK activation and hypertrophy signalling. Novel proteins, not previously associated with resynchronization, are presented; and protein–protein interaction and pathophysiological relevance of these findings can be studied in a genetically modifiable dyssynchrony and resynchronization model for the first time.

## Conflict of interest

Hugo Nordin, Ryo Nakagawa, Marita Wallin, John Pernow, David A. Kass, and Marcus Ståhlberg declare that they have no conflict of interest.

## References

- Cleland JG, Daubert JC, Erdmann E, Freemantle N, Gras D, Kappenberger L, Tavazzi L. The effect of cardiac resynchronization on morbidity and mortality in heart failure. *N Engl J Med* 2005; **352**: 1539–1549.
- Goldenberg I, Kutiyafa V, Klein HU, Cannom DS, Brown MW, Dan A, Daubert JP, Estes NA 3rd, Foster E, Greenberg H, Kautzner J, Klempfner R, Kuniss M, Merkely B, Pfeffer MA, Quesada A, Viskin S, McNitt S, Polonsky B, Ghanem A, Solomon SD, Wilber D, Zareba W, Moss AJ. Survival with cardiac-resynchronization therapy in mild heart failure. *N Engl J Med* 2014; **370**: 1694–1701.
- Vernooy K, Cornelussen RN, Verbeek XA, Vanagt WY, van Hunnik A, Kuiper M, Arts T, Crijns HJ, Prinzen FW. Cardiac resynchronization therapy cures dyssynchronopathy in canine left bundle-branch block hearts. *Eur Heart J* 2007; **28**: 2148–2155.
- Prinzen FW, Hunter WC, Wyman BT, McVeigh ER. Mapping of regional myocardial strain and work during ventricular pacing: experimental study using magnetic resonance imaging tagging. *J Am Coll Cardiol* 1999; **33**: 1735–1742.
- Prinzen FW, Augustijn CH, Arts T, Allesie MA, Reneman RS. Redistribution of myocardial fiber strain and blood flow by asynchronous activation. *Am J Physiol* 1990; **259**: H300–H308.
- Prinzen FW, Cheriex EC, Delhaas T, van Oosterhout MF, Arts T, Wellens HJ, Reneman RS. Asymmetric thickness of the left ventricular wall resulting from asynchronous electric activation: a study in dogs with ventricular pacing and in patients with left bundle branch block. *Am Heart J* 1995; **130**: 1045–1053.
- Vernooy K, Verbeek XA, Peschar M, Crijns HJ, Arts T, Cornelussen RN, Prinzen FW. Left bundle branch block induces ventricular remodelling and functional septal hypoperfusion. *Eur Heart J* 2005; **26**: 91–98.
- Das DK, Maulik N, Moraru II. Gene expression in acute myocardial stress. Induction by hypoxia, ischemia, reperfusion, hyperthermia and oxidative stress. *J Mol Cell Cardiol* 1995; **27**: 181–193.
- Kubisch C, Wollnik B, Maass A, Meyer R, Vetter H, Neyses L. Immediate-early gene induction by repetitive mechanical but not electrical activity in adult rat cardiomyocytes. *FEBS Lett* 1993; **335**: 37–40.
- Barth AS, Aiba T, Halperin V, DiSilvestre D, Chakir K, Colantuoni C, Tunin RS, Dimaano VL, Yu W, Abraham TP, Kass DA, Tomaselli GF. Cardiac resynchronization therapy corrects dyssynchrony-induced regional gene expression changes on a genomic level. *Circ Cardiovasc Genet* 2009; **2**: 371–378.
- Stahlberg M, Nakagawa R, Bedja D, Zhu G, Lin BL, Saberi A, Lee DI, Kass DA. Chronic atrial and ventricular pacing in the mouse. *Circ Heart Fail* 2019; **12**: e005655.
- Epskamp S, Cramer A, Waldorp LJ, Schmittmann VD, Borsboom D. qgraph: network visualizations of relationships in psychometric data. *J Stat Softw* 2012; **48**: 1–18.
- Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, Simonovic M, Doncheva NT, Morris JH, Bork P, Jensen LJ, Mering CV. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res* 2019; **47**: D607–D613.
- Chakir K, Daya SK, Tunin RS, Helm RH, Byrne MJ, Dimaano VL, Lardo AC, Abraham TP, Tomaselli GF, Kass DA. Reversal of global apoptosis and regional stress kinase activation by cardiac resynchronization. *Circulation* 2008; **117**: 1369–1377.
- Kirk JA, Holewinski RJ, Kooij V, Agnetti G, Tunin RS, Witayavanitkul N, de Tombe PP, Gao WD, Van Eyk J, Kass DA. Cardiac resynchronization sensitizes the sarcomere to calcium by reactivating GSK-3beta. *J Clin Invest* 2014; **124**: 129–138.