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# Targeted DNA methylation profiling of human cardiac tissue reveals novel epigenetic traits and gene deregulation across different Heart Failure patient sub-types

**Glezeva**

**DNA methylation profiling of Heart Failure**

Nadezhda Glezeva, PhD [1, 2, 3], Bruce Moran, PhD [1], Patrick Collier, PhD [4], Christine S. Moravec, PhD [5], Dermot Phelan, PhD [4], Eoin Donnellan, MD [4], Adam Russell-Hallinan, BSc [1], Darran P. O'Connor, PhD [6], William M. Gallagher, PhD [1], Joe Gallagher, MD [2], Kenneth McDonald, MD [1, 2, 3], Mark Ledwidge, PhD [1, 2, 3], John Baugh, PhD [1], Sudipto Das, PhD [6]†, Chris J. Watson, PhD [1, 3, 7]†*

[1] UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Ireland

[2] Heart Failure Unit, St. Vincent's University Hospital Healthcare Group, Elm Park, Dublin, Ireland

[3] The Heartbeat Trust, Dun Laoghaire, Dublin, Ireland

[4] Department of Cardiovascular Medicine, Cleveland Clinic, Cleveland, Ohio 44195, USA

[5] Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA

[6] Department of Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, Dublin, Ireland

[7] The Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Northern Ireland

† Equal contribution

* Corresponding author:

Dr. Chris J. Watson,

Centre for Experimental Medicine

Wellcome-Wolfson Institute for Experimental Medicine

Queen's University Belfast

Belfast BT9 7BL

Northern Ireland

E-mail: chris.watson@qub.ac.uk

Tel: +44 2890976478

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## Abstract

**BACKGROUND:** Limited knowledge exists of the extent of epigenetic alterations, such as DNA methylation, in Heart Failure (HF). We conducted targeted DNA methylation sequencing to identify DNA methylation alterations in coding and non-coding RNA across different etiological sub-types of HF.

**METHODS AND RESULTS:** A targeted bisulfite sequence capture sequencing platform was applied to DNA extracted from cardiac interventricular septal tissue of 30 male HF patients encompassing etiologies including Hypertrophic Obstructive Cardiomyopathy (HOCM), Ischemic Cardiomyopathy (ISCM), Dilated Cardiomyopathy (DCM), and 9 control patients with non-failing hearts (NF). We detected 62,678 differentially methylated regions (DMR) in the studied HF cohort. By comparing each HF sub-group to the NF control group we identified 195 unique DMRs: 5 in HOCM, 151 in DCM, and 55 in ISCM. These translated to 4 genes/1 non-coding RNA in HOCM, 131 genes/17 non-coding RNA in DCM, and 51 genes/5 non-coding RNA in ISCM. Subsequent gene/non-coding RNA expression analysis was assessed using qRT-PCR and revealed 6 genes: 4 hypermethylated (*HEY2*, *MSR1*, *MYOM3*, *COX17*), 2 hypomethylated (*CTGF*, *MMP2*); and 2 microRNA: 1 hypermethylated (miR-24-1), 1 hypomethylated (miR-155) with significantly up- or down-regulated expression levels consistent with the direction of methylation in the particular HF sub-group.

**CONCLUSIONS:** For the first time DNA methylation alterations and associated gene expression changes were identified in etiologically-variant pathological heart failure tissue. The methylation-sensitive and disease-associated genes/non-coding RNA identified from this study represent a unique cohort of loci that demonstrate a plausible potential as a novel diagnostic and/or therapeutic target in HF and warrant further investigation.

## Introduction

Important pathological mechanisms that regulate gene expression involve epigenetic modifications such as methylation of DNA and non-coding RNA (ncRNA). Several comprehensive reports have suggested their plausible role in HF pathogenesis [1, 2]. Specifically, DNA methylation is a unique epigenetic process for fine-tuning of gene expression in line with the needs of the body and in response to the ever-changing environmental milieu [5]. This process is conserved and commonly linked to transcriptional gene repression as it can prevent binding of transcription factors to the DNA or limit the access to gene regulatory regions.

Aberrant patterns of DNA methylation have been shown to contribute to maladaptive cardiac remodeling including hypertrophy, fibrosis, ischemia, and inflammation, as well as to occur as a consequence of these disease processes [6]. To date, studies that have assessed DNA methylation profiling in HF patients have mostly used whole-genome bisulfite sequencing to assess global changes in methylation and epigenomic patterns in blood or cardiac tissue from patients from a single HF etiology (end—stage ischemic/idiopathic HF, dilated cardiomyopathy, ischemic cardiomyopathy) compared to a non-HF control group. However, no study has so far examined DNA methylation signatures in other significant HF etiologies such as hypertrophic cardiomyopathy (HCM), in particular obstructive HCM (HOCM), and no study has used a targeted methylated capture approach to examine DNA methylation in genes associated specifically with the HF syndrome. Such methylation signatures could be used to discover novel diagnostic and therapeutic targets for HF.

## Methods

Custom-designed Next Generation targeted methylation sequencing was performed in left ventricular septal tissues of 39 male patients who underwent orthotropic cardiac transplantation for ischemic cardiomyopathy (ISCM, 9), dilated cardiomyopathy (DCM, 9), or septal myectomy for hypertrophic cardiomyopathy (HOCM, 12), and in matched control patients with non-failing hearts (NF, 9) who died of non-cardiac causes. See Supplemental Material for a detailed description of the Methods.

# Results

## Clinical classification of the studied patient cohort

Characteristics of the studied patient cohort are listed in Supplemental Table 1. There was no statistically-significant difference in age, gender, and body mass index between the groups.

## Altered DNA methylation in HF patients

A total of 62,678 500bp-long differentially methylated regions (DMRs) were analyzed for altered methylation in interventricular septal tissue. A difference in methylation of ≥10% at 5x coverage with 5% FDR in each HF patient group when compared to the NF control group were considered for further analysis. We identified 195 unique DMRs in the HF cohorts versus control: 5 in HOCM, 151 in DCM, and 55 in ISCM patients.

Non-negative matrix factorization (NMF) clustering (fig. 1A) demonstrates subtle differences between HF subgroups. Such findings were expected considering that analyzed tissues were sourced from the left ventricular (LV) septum, and that the studied cohort consisted of HF patients who, despite differences in etiology, have common cardiac remodeling features. This is in contrast to other disease types such as cancer where big methylation differences are expected and evident. NMF clustering allowed a distinctive separation of the HOCM cohort, and to some degree in the DCM group, which had the greater number of identified DMRs. This was further supported by the PCA plots (Supplemental fig. 1) which indicated that patient samples from different HF disease groups are not highly divergent in the first two principal components but do cluster/separate as expected.

The identified regions were next annotated against known protein-coding genes and ncRNA and subdivided into regions with increased (hypermethylated) and reduced (hypomethylated) methylation (fig. 1B). In the HOCM patient group, 4 protein-coding genes (3 hypermethylated, 1 hypomethylated) and 1 ncRNA (1 hypomethylated) were found to be differentially methylated. The DCM group was most divergent with 131 protein-coding genes (13 hypermethylated, 118 hypomethylated) and 17 ncRNA (3 hypermethylated, 14 hypomethylated) identified as having altered methylation profiles. In

ISCM patients, 51 protein-coding genes (8 hypermethylated, 43 hypomethylated) and 5 ncRNA (3 hypermethylated, 2 hypomethylated) were differentially methylated. Venn diagrams were created to illustrate protein-coding genes and ncRNA which were methylated in ≥1 patient group(s) (fig. 1C).

## Aberrant DNA methylation regulates protein-coding gene and non-coding RNA expression in HF patients

To examine the impact of DNA methylation alterations at specific loci on gene expression, qRT-PCR analysis was performed on a subset of 28 protein-coding genes and 5 micro RNA (miRNA), which exhibited differential DNA promoter methylation in ≥1 of the studied HF groups (Supplemental Table 2). qRT-PCR was performed for all 39 patients. The 28 coding/non-coding RNA were selected based on several criteria described in Supplemental Material.

The analysis showed significantly altered expression of 6 protein-coding genes and 2 miRNA in ≥1 HF groups (Table 1). *In silico* analysis of the specific methylated regions identified in the putative promoters (-2000/+500 bp from the transcriptional start site) of these coding/non-coding RNA revealed that these sites contain active transcription marks including H3K27ac, H3K9ac, H3K27me3, as well as DNaseI hypersensitivity clusters (UCSC genome browser). This supports the fact that the methylation alterations at such potential regulatory regions could plausibly impact gene expression across the various sample types.

Within this context, of the identified 6 protein-coding genes with significantly altered gene expression, 4 were hypermethylated (*HEY2, MSR1, MYOM3, COX17*) and 2 were hypomethylated (*CTGF*, *MMP2*). Moreover, the patterns of gene expression were consistent with the direction of DNA methylation., i.e. genes with hypermethylated promoters incurred reduced gene expression compared to the NF group, whereas those with hypomethylated promoters had increased gene levels.

Amongst the 4 hypermethylated protein-coding genes, *HEY2* and *MSR1* were significantly hypermethylated in HOCM with gene expression significantly reduced by 0.53-fold and 0.42-fold, respectively, in HOCM versus the NF control group.. *MYOM3* and *COX17* were hypermethylated in ISCM, and their transcript levels were significantly reduced by 0.74-fold and 0.49-fold, respectively.

Of the 2 hypomethylated protein-coding genes *MMP2* was significantly hypomethylated in DCM, and *CTGF* – in ISCM and DCM at two neighboring DMR (Table 1). Expression levels of *MMP2* were increased by 2.67-fold in DCM, and *CTGF* was upregulated by 3.33-fold in ISCM and 2.85-fold in DCM.

From a ncRNA perspective, DNA methylation analysis showed the miR-23b/miR-27b/miR24-1 cluster to be significantly hypermethylated in ISCM and DCM at two different regions, and miR-155 to be hypomethylated in ISCM. Whilst we did not find significant differences in the expression of miR-23b or miR-27b (data not shown), expression of miR24-1 was significantly reduced by 0.81-fold in the ISCM group only. MiR-155 expression was increased by 1.63-fold in ISCM patients, tandem with its hypomethylation profile in this group.

## Discussion

DNA methylation alterations aredynamic epigenetic modifications, leading to a widespread regulation of gene expression. Despite the vast clinical potential of DNA methylation sequencing not many studies to date have used this technique to identify disease-associated methylation changes in HF patients. In this study we performed targeted DNA methylation profiling to identify patterns characteristic for HOCM, DCM, and ISCM etiologies.

We report 5 novel significantly-expressed differentially-methylated regions in HOCM, 151 in DCM, and 55 in ISCM patients.

The identified novel DMRs were found to associate with the promoter regions of a number of protein-coding genes and ncRNA, some of which have been shown to regulate key processes in HF underlined by hypertrophic, dilated, or ischemic cardiomyopathy. We also identified 2 novel HOCM-related genes, *HEY2* and *MSR1*, which were deregulated in disease. We identified significant hypermethylation in *HEY2* and *MSR1* complemented with considerably reduced expression of these genes in HOCM tissue. *HEY2* is an important effector in the Notch developmental pathway with a critical role in heart development, and studies have demonstrated *HEY2* mutations and deletions in patients with atrioventricular septal defects [5] and congenital heart defects [6]. Our finding of reduced

*HEY2* expression likely due to increased methylation in HOCM patients is a novel discovery highlighting DNA methylation as an additional mechanism of *HEY2* silencing in HCM. Likewise, we report novel hypermethylation and aberrant expression of *MSR1* in HOCM. *MSR1* is a macrophage-restricted gene responsible for optimized inflammatory response and lipid homeostasis and its deletion has been reported to cause cardiac rupture following an experimental ischemic insult (MI) [7]. Thus, our study demonstrates DNA hypermethylation as a likely regulatory mechanism that controls expression of these two genes novel to HOCM.

From the methylation analysis in DCM tissue, we report hypomethylation with significantly elevated gene expression of two genes important for the turnover and stability of the extracellular matrix (ECM) – *MMP2* and *CTGF*. *MMP2* is a well-known regulator of collagen turnover and fibrosis and we and others have reported higher levels of this protein in peripheral serum and heart tissue of patients at risk for or with established HF [8-10] including HF due to DCM. Likewise, *CTGF* is primarily implicated in ECM fibrosis in DCM and is highly expressed in failing hearts [9]; moreover CTGF inhibition has been shown to significantly improve LV function and slow the progression of LV dilatation in a mouse model of DCM [11]. So far, cardiac studies have been limited in providing a mechanistic insight into the regulation of *MMP2* and *CTGF* in HF and our study for the first time reports methylation as a likely mechanism for gene regulation.

Analysis of ISCM tissue revealed hypermethylation and diminished gene expression of *MYOM3* and *COX17*, and hypomethylation of *CTGF* with elevated gene expression. *MYOM3* is one of three myomesin genes which have complex roles in muscle contractility and myofibril assembly. *COX17* is a copper chaperone for cytochrome c oxidase and a highly conserved protein in the mitochondrial respiratory chain. It is implicated in several processes including heart development and its deficiency is associated with end-stage DCM [12]. With relation to hypomethylated genes, we identified significant hypomethylation of *CTGF* in ISCM patients at a unique region, different to the DMR identified in the DCM group. Expression levels of *CTGF* were 3-times higher than in the control group. Similar findings of increased *CTGF* expression in LV tissue of ISCM patients were previously reported [9, 13] and authors gave evidence of correlation of *CTGF* to genes involved in ECM remodeling [13] and

localization of *CTGF* to regions of fibrosis in the failing heart [9]. In addition, *CTGF* promoter hypomethylation was reported to augment hepatic fibrosis by promoting phenotypic changes of hepatic stellate cells into myofibroblasts [14]. Alongside published data, our data suggests that regulation of *CTGF* by DNA methylation promotes adverse cardiac remodeling in the heart of ISCM patients.

From a ncRNA perspective in ISCM, we identified hypermethylation in 2 regions relating to the miR-23b/miR-27b/miR-24-1 cluster and confirmed reduced expression specifically of miR-24-1. We also found significant hypomethylation and increased expression of miR-155 in ISCM. Though the significance of the miR-23b/miR-27b/miR-24-cluster in vascular remodeling (particularly neovascularization, atherosclerosis and aneurysm) has been discussed [15], there is no evidence linking it, or linking miR-24-1 to HF or ISCM. Similarly, miR-155 is a well-characterized miRNA within the context of cardiovascular remodeling and disease [15, 16], however there have been only few studies investigating its role in HF. One recent study assessed the value of several miRNAs to serve as circulating biomarkers of disease and reported increased miR-155 expression in coronary sinus samples from patients with congestive HF [17]. However, our study is the first to show altered miR-155 expression within human cardiac tissue of patients with heart failure thus highlighting a potential new implication for this miRNA in HF pathogenesis.

In summary, this study provides an insight into the DNA methylation landscape of cardiac tissue from HF patients presenting with varied clinical etiologies (HOCM, DCM, ISCM), and supports the role of DNA methylation in regulation of disease-associated genes. This study presents an avenue to carry out an additional analysis into the functional role of the methylation-sensitive genes identified herein in order to determine their precise mechanistic role in HF. Further investigation of the cell type-specific methylation patterns of these methylated protein-coding genes and ncRNA will provide valuable information of the cardiac cell types (cardiomyocytes, fibroblasts, inflammatory cells, endothelial cells) involved in the methylation process, that either drives HF pathogenesis or occurs as a result of it. This proposed analysis would indeed account for the varied cellular composition that may be seen in some HF pathologies.

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## Disclosures

None.

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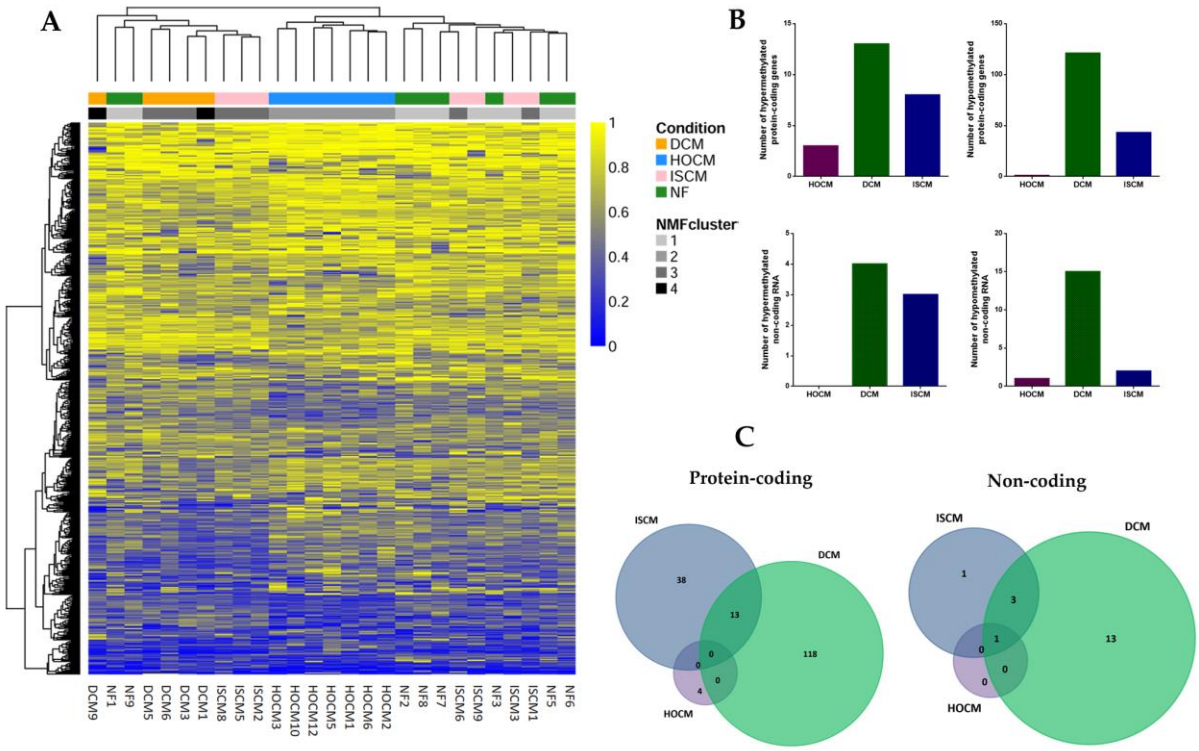
## Figure Legends

**Figure 1** DNA methylation of protein-coding genes and non-coding RNA that were significantly modulated in the studied HF patient cohort. **A)** Heatmap showing non-negative matrix factorization clustering of methylation profiles of NF Control, HOCM, DCM, and ISCM groups. The degree of methylation in each patient at n=690 500bp tiles is presented from 0% (0, blue) to 100% (1, yellow). **B)** Bar graphs illustrating the number of hyper- and hypo-methylated protein-coding genes and non-coding RNA in HOCM, DCM, and ISCM groups as compared to the control, NF group. Differential hypomethylation of promoter regions is prominent in all 3 groups. C**)** Venn diagrams illustrating differential methylation profiles of HOCM, DCM, and ISCM as compared to NF control, in terms of the number of protein-coding genes (left) and non-coding RNA (miRNA and long non-coding RNA, right) involved. Methylation events specific to 1 and >1 patient group are shown. HOCM is depicted in purple colour, DCM – in green, ISCM – in blue.



## Tables

**Table 1** Methylation and expression levels of selected protein-coding genes and miRNA linked to methylated DMR in HF patient groups versus NF controls

| Gene / miRNA | Direction of methylation | Chromosomal coordinates of methylated DMR | Regulatory function of methylated DMR | HF patient group where significant methylation identified | % Methylation difference vs. NF control group | P-FDR | Fold gene / miRNA expression vs. NF control group | P-value |
|---|---|---|---|---|---|---|---|---|
| ***HEY2*** | hypermethylated | chr6: 126,067,001-126,067,501 | promoter | HOCM | 15.81 | 0.006 | 0.53 | 0.001 |
| ***MSR1*** | hypermethylated | chr8:15,964,501-15,965,001 | none | HOCM | 19.87 | 0.044 | 0.42 | 0.003 |
| ***COX17*** | hypermethylated | chr3:119,373,001-119,373,501 | none | ISCM | 25.99 | 0.046 | 0.49 | 0.001 |
| ***MYOM3*** | hypermethylated | chr1:24,380,501-24,381,001 | none | ISCM | 21.25 | 0.003 | 0.74 | 0.019 |
| ***miR24-1*** [§] | hypermethylated | chr9:97,847,001-97,847,501 | H3K27Ac (active regulatory region) | ISCM | 11.27 | 0.035 | 0.81 | 0.031 |
| ***CTGF*** | hypomethylated | chr6:132,268,501-132,269,001 | H3K27Ac (active regulatory region) | ISCM | 17.52 | 0.00003 | 2.85 | 0.005 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | hypomethylated | chr6:132,268,001-132,268,501 | H3K27Ac (active regulatory region) | DCM | 11.42 | 0.019 | 3.33 | 0.011 |
| ***MMP2*** | hypomethylated | chr16:55,511,001-55,511,501 | none | DCM | 14.45 | 0.032 | 2.67 | 0.003 |
| ***miR155*** | hypomethylated | chr21:26,946,001-26,946,501 | H3K27Ac (active regulatory region) | ISCM | 16.41 | 0.005 | 1.63 | 0.030 |

p-FDR, False Discovery Rate corrected p-value; DMR, differentially methylated region; [§] miR24-1 hypermethylation is identified as part of the miR23b/miR27b/miR24-1 cluster