RNA-seq profiling reveals different pathways between remodeled vessels and myocardium in hypertrophic cardiomyopathy

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Abstract

Objective: Coronary microvascular dysfunction (CMD) is a key pathophysiological feature of hypertrophic cardiomyopathy (HCM), contributing to myocardial ischemia and representing a critical determinant of patients’ adverse outcome. The molecular mechanisms underlying the morphological and functional changes of CMD are still unknown. Aim of this study was to obtain insights on the molecular pathways associated with microvessel remodeling in HCM.

Methods: Interventricular septum myectomies from patients with obstructive HCM (n = 20) and donors’ hearts (CTRL, discarded for technical reasons, n = 7) were collected. Remodeled intramyocardial arterioles and cardiomyocytes were microdissected by laser capture and next-generation sequencing was used to delineate the transcriptome profile.

Abbreviations: BP, biological processes; CC, cellular compartment; CMD, coronary microvascular dysfunction; DEGs, differentially expressed genes; FDR, false discovery rate; GO, gene ontology; HCM, hypertrophic cardiomyopathy; HE, hematoxylin-eosin; LA, lumen area; LCM, laser capture microdissection; LVH, left ventricular hypertrophy; MF, molecular functions; NGS, next-generation sequencing; RIN, RNA integrity number; SEM, standard error of the mean; VA, vessel area.

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1 | INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most common genetic heart disease, with a prevalence of 1:500 in the general population and is mainly caused by single-gene mutations in genes encoding proteins of the sarcomere. The mutations lead to structurally and functionally altered proteins, generating a cascade of secondary defects in cardiomyocyte energetics, contractility, and structure.

Macroscopically HCM demonstrates marked left ventricular hypertrophy (LVH), often asymmetric, and ventricular dysfunction. Microscopically, it is characterized by cardiomyocyte hypertrophy and heterogeneously distributed spatial disarray, interstitial fibrosis and adverse remodeling of intramural coronary arterioles (i.e., vessel wall thickening with hypertrophy of smooth muscle cells and increased collagen deposition in the tunica media with variable degrees of intimal thickening) with local ischemia.

Coronary microvascular dysfunction (CMD) represents a key pathophysiological mechanism in HCM, contributing to myocardial ischemia and replacement fibrosis, pointing to CMD as a critical determinant of adverse outcome in HCM. Whether CMD is part of the same gene deregulation associated to myocardial alteration is unknown.

In the last decades, the development of laser capture microdissection (LCM) allowed high-resolution isolation of selected cells/tissue portions from tissue sections, preserving the molecular composition for omics analyses. Recently, the coupling of LCM with next-generation sequencing (NGS) has been proposed as powerful strategy to investigate and compare the transcriptome profiles of different components of the same samples, but low quantities of starting RNA can be a severe hindrance, especially for RNA-sequencing studies. Multiple protocols have been developed for transcriptome profiling from very low-quantity RNA inputs and recent advances in RNA-sequencing technology enable sequencing analysis with limited amounts of RNA obtained from selected areas of interest.

In the current study, we applied the combined approach of LCM and full-length mRNA-sequencing to compare the transcriptome profiling of remodeled arterioles and cardiomyocytes from interventricular septal tissue of HCM patients vs. controls (CTRL, donor hearts). Differentially expressed genes (DEGs) were identified and analyzed by comparative functional enrichment to obtain insights on the pathways putatively associated with HCM and related either to cardiomyocyte alterations or CMD.

2 | MATERIALS AND METHODS

2.1 | Sample collection

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and conformed to Sapienza University of Rome Ethical Committee protocols.

Myocardial samples were obtained from patients with obstructive HCM, diagnosed according to current guidelines, undergoing septal myectomy procedures at Careggi University Hospital, Florence (n = 4) and San Raffaele Hospital, Milan (n = 16). All patients gave informed consent for the procedure.

Control myocardial samples (CTRL, n = 7) from the same site of the septal myectomy procedure (subaortic septum) were collected at Sapienza University Hospital in Rome from donors’ hearts discarded from transplantation because of noncardiac technical reasons (e.g., suitable recipient unavailability).

Samples were harvested immediately after surgery and cut into 1-μm thick slices perpendicular to the endocardium. Part of each specimen was embedded in KilliK (O.C.T. BioOptica) and snap-frozen in nitrogen-cooled isopentane for in situ gene-expression studies. The remaining tissue was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological and histomorphometric analyses.

2.2 | Histological and histomorphometric analysis

Eight-μm-thick sections were obtained from each paraffin block, de-paraffinized, rehydrated and stained with hematoxylin-eosin.
(HE) and Azan Mallory stain for light microscopy. For the purpose of the study, small vessels were defined as intramural arterioles with a diameter <100 μm. For each case, the small vessel medial hypertrophy and perivascular fibrosis, as well as the presence of myocardial hypertrophy, myofiber disarray, interstitial fibrosis, and myocardial microscarring were recorded. High-resolution images of intramural arterioles were acquired at 20x magnification with a digital camera (Olympus). Images were stored as TIF files and were analyzed by a dedicated software (ImageJ 1.47v, Wayne Rasband National Institutes of Health). Lumen area (LA) and vessel area (VA) were manually measured. The following parameters were then derived: Medial area (VA-LA); Lumen area to vessel area ratio (LA/VA); Lumen diameter [√(LA/3.14)] × 2 and Vessel diameter [√(V/3.14)] × 2 as previously published.

2.3 | Statistical analysis

For the histomorphometric analysis, all data are reported as mean ± standard error of the mean (SEM). For direct comparison between HCM patients and controls, unpaired Student’s t test was used. Significance was considered at p < .05. Numerical estimates were obtained with the GraphPad Prism 7 version (GraphPad Inc).

2.4 | LCM procedure

Frozen sections from the myectomy specimens with a thickness of 10 μm were mounted onto PEN-membrane glass slides (Leica Microsystems) and stained with HE. Between 100–150 remodeled intramyocardial arterioles were microdissected by laser capture with the Leica LMD 7000 (Leica Microsystems). For each sample, similar amounts of dissected cardiomyocytes were also obtained (Figure S1).

2.5 | RNA preparation

Total RNA from microdissected samples was isolated by miRNAeasy Micro Kit (Qiagen) specific for purifying total RNA from small amounts of tissue. The concentration and purity of total RNA were determined using Agilent RNA 6000 Pico Assay (Agilent 2100 Bioanalyzer). Only the samples presenting RNA Integrity Number (RIN) ≥ 7 were selected for the further next-generation sequencing study.

2.6 | RNA-sequencing

RNA was converted into RNA-seq libraries with the Clontech Smarter kit (specifically designed for RNA-Seq applications involving laser-captured samples) and sequenced on an Illumina Nextseq 500 sequencer with a HighOutput flow cell, 1 × 75 nt, single-end reads.

2.7 | RNA-seq data analysis

2.7.1 | Pre-processing

Quality control for 75-base single-end reads of each sample was performed by using the FastQC tool, which can examine sequence quality, GC content, presence of adapters, over-represented k-mers, and read duplication. The Trimmmomatic software (v. 0.36) was used to discard low-quality reads (average quality < 28), eliminate poor-quality bases from their 3’ end, and trim adapter sequences. Only reads longer than 35 bases were retained and mapped on the human reference genome (GRCh38) by using HISAT2 aligner (v2.1.0) with default parameter values.

2.7.2 | Differential expression analysis (HCM versus CTRL) in myocardium and vessel tissue

Read counts for each human gene were estimated by using StringTie software (v2.1.1) with the human transcriptome from Ensembl (release 98) as reference annotation, followed by running the prepDE.py Python script to generate the count matrix (as provided and suggested in the StringTie protocol). Mitochondrial genes were excluded from the analysis to avoid the introduction of significant biases in the differential expression analysis due to their high expression levels in the myocardium. Principal Component Analysis (PCA) and correlation coefficient analysis were performed to examine gene-expression level of HCM and CTRL samples both in cardiomyocytes and vessels, assessing similarities and differences between groups. Normalization and differential expression test were performed using DESeq2 R-package (v1.26). For each comparison, only genes with more than 1 count per million (cpm) in a minimum number of samples (the size of the group with the lowest number of samples under analysis) were retained. Multiple-testing correction to control the false discovery rate (FDR) was performed by applying Benjamin–Hochberg method. Only the genes with an adjusted p < .01 were marked as differentially expressed genes (DEGs) (significantly up- and down-regulated). As the biological importance of a given change in expression level is unknown, no fold-change cutoff was applied. Genes that were differentially expressed (adjusted p < .01) between HCM versus CTRL in myocardium, but not in vessels (adjusted p > .01), were marked as exclusive myocardium-DEGs, and vice versa for exclusive vessel-DEGs. Gene overlaps were calculated using the InteractiVenn software.

2.8 | Functional enrichment analysis

A functional enrichment analysis was performed on each set of differentially expressed (protein-coding) genes between HCM and CTRL, both in myocardium and arterioles, keeping the up- and down-regulated groups. Over-represented biological processes, functions, or pathways (terms which have more DEGs
than expected by chance) were identified by using DAVID web-
server, with the entire human proteome as reference and
querying the following functional categories: Gene Ontology (GO)
terms related to Biological Processes (BP), Molecular Functions
(MF), and Cellular Compartment (CC); protein families as classi-
fied by InterPro database; pathways collected in KEGG and
Reactome databases; UniProt protein annotations, and pu-
tative molecular interactors as annotated in IntAct database. Only
biological categories with Benjamini–Hochberg corrected
p-value (adjusted p-value ≥ 5 × 10^-2) were considered as sta-
tistically enriched. Results for each investigated group (up-
and down-regulated protein-coding genes, both in myocardium and
vessel) are shown as heatmaps, with the color scale representing
the adjusted p-values, created using the gplots R-package (https://
CRAN.R-project.org/package=gplots).

3 | RESULTS

3.1 | Study population

Twenty patients with a diagnosis of obstructive HCM according to
current guidelines were enrolled. Baseline clinical and echocardi-
ographic data are summarized in Table 1. Seven CTRL biopsies were
collected. Due to privacy law, only data regarding age and sex of
donors were available. The control group consisted of 4 males and 3
females, with a mean age of 55 ± 8 years.

3.2 | Histologic and morphometric features

Histologic evaluation of myectomy samples was in keeping with the
clinical diagnosis of HCM, showing myocyte hypertrophy and areas
of myofiber disarray, characterized by bundles of myocytes crossing
each other with a herringbone pattern. Microscopic examination
also revealed the presence of both interstitial and replacement
fibrosis, the latter frequently surrounding remodeled coronary ar-
terioles, with medial wall thickening, mainly due to smooth muscle
hypertrophy and increased collagen deposition and variable intimal
thickening. Histologic analysis of control samples showed a normal
myocardial and vessel structure (Figure S2).

Morphometric analysis showed a significant increase in micro-
vascular medial area in HCM samples as compared to CTRL,
(9578.59 ± 1295.76 μm² vs 3752.03 ± 536.7 μm², p = .0001) paral-
leled by a decrease of the lumen-to-vessel area ratio (0.10 ± 0.01 vs
0.24 ± 0.02 in HCM patients and controls, p = .0001).

3.3 | Detection of differentially expressed genes in HCM versus CTRL, common and specific to

myocardium and vessel tissues

NGS was used to delineate the transcriptome profile of car-
diomyocytes and arterioles in HCM and CTRL samples. For these

| TABLE 1 Baseline characteristics and echocardiographic data of HCM patients |
|-------------------------------|------------|
| **Patients**                  | **N = 20** |
| **Demographic data**          |            |
| Age (years), M (SD)           | 59 (8)     |
| Male gender, N (%)            | 14 (70)    |
| BMI (kg/m²), M (SD)           | 27 (5)     |
| **Clinical data**             |            |
| Positive genetic screening, N (%) | 7 (35) |
| VUS, N (%)                    | 3 (15)     |
| Family history of HCM, N (%)  | 7 (35)     |
| NYHA class ≥ III, N (%)       | 8 (40)     |
| Angina, N (%)                 | 2 (10)     |
| Syncope, N (%)                | 4 (20)     |
| NSVT, N (%)                   | 1 (5)      |
| **Medical therapy**           |            |
| Beta-blockers, N (%)          | 18 (90)    |
| Antiarrhythmic drugs, N (%)   | 4 (20)     |
| Diuretics, N (%)              | 12 (60)    |
| RAAS-i, N (%)                 | 12 (60)    |
| CCB, N (%)                    | 3 (15)     |
| **Echocardiographic data**    |            |
| IVS thickness (mm), M (SD)    | 22 (5)     |
| LV-EDV (ml), M (SD)           | 141 (62)   |
| LV-EF (%), M (SD)             | 67 (9)     |
| Moderate-to-severe mitral regurgitation, % | 85 |
| SAM-related LVOT-max gradient at rest (mmHg), M (SD) | 68 (35) |

Abbreviations: aldosterone system inhibitors; angiotensin-1, BMI, body
mass index; CCB, calcium channel blockers; EDV, end diastolic volume;
EF, ejection fraction; IVE, interventricular septum; LV, left ventricular;
LVOT, left ventricular outflow tract; M, mean; Max, maximum; N,
number; NSVT, non-sustained ventricular tachycardia; NYHA class,
New York Heart Association class; RAAS-i, renin-SD, standard
deviation; VUS, variants of uncertain significance.

experiments, we dissected arterioles from 20 HCM hearts and 6
CTRL, and cardiomyocytes from 10 HCM and 5 CTRL, selected for
RNA quality (RIN > 7).

Reads univocally mapped on the human reference genome
(>70% of the sequenced reads) were used to estimate the gene-
expression values in all samples (Figures S3 and S4). Exploratory
gene-expression PCA plots (with respect to the first two compo-
nents) visualized the distribution of the samples (Figures S5 and S6),
showing an overall separation between HCM and CTRL. The higher
dispersion of CTRL revealed more heterogeneous expression with
respect to clustered HCM points, indicative of inherently greater
variability in the healthy CTRL than in HCM patients.

The Volcano Plots (Figure 1) showed a balanced (symmetrical)
distribution of data, revealing no alterations in the results due to bias
or artifacts between HCM and CTRL.

Our technique allowed the detection of a high number of genes
differentially expressed between HCM and normal heart, both in
cardiomyocytes and in remodeled arterioles. In fact, a total of 890 differentially expressed genes (DEGs) were detected in cardiomyocytes (387 DEGs up- and 503 DEGs down-regulated) and 1485 DEGs were identified in the remodeled arterioles (675 up-regulated and 810 down-regulated, Tables S1 and S2).

Among the differentially expressed genes, 170 were altered both in HCM cardiomyocytes and arterioles. Of those, 25 were parallel up-regulated and 142 down-regulated (Figure 2). Conversely, two genes, the C-type lectin domain-containing 16A (CLEC16A) and Myeloid/lymphoid or mixed-lineage leukemia translocated to, 11 (MLLT11, an inducer of bad-mediated intrinsic apoptosis), were up-regulated in cardiomyocytes and down-regulated in arterioles. Only one gene, the IQ domain-containing protein N (IQCN), was down-regulated in cardiomyocytes and up-regulated in arterioles.

Interestingly, we were able to identify "exclusive DEGs" either for cardiomyocytes or arterioles, that is, DEGs between HCM and control in cardiomyocyte but not in remodeled arterioles samples and vice versa. Moreover, we found that the vast majority of DEGs are indeed exclusive: 360 (out of 387) and 360 (out of 503) up- and down-regulated, respectively, in cardiomyocytes, and 649 (out of 675) and 666 (out of 810) up- and down-regulated, respectively, in arterioles (Figure 2, Table 2 and Tables S3 and S4).

3.4 Comparative functional enrichment analyses identify tissue-specific pathways potentially altered in CMD

To detect DEGs potentially involved in HCM pathogenesis, we performed Gene Ontology and Pathway enrichment analyses, two fundamental investigations exploring expression data. More specifically, comparative functional analyses were performed across up- and down-regulated DEGs in HCM cardiomyocytes and arterioles by using Gene Ontology terms (BP, CC, and MF), KEGG and Reactome Pathways, InterPro domains, UniProt protein annotations, and the IntAct molecular interaction annotations (Figures S7–S10).

Enriched biological processes and pathways included terms such as proteasome (GO0005839, hsa03050), apoptosis (R-HSA-109581), PI3K activates AKT signaling (R-HSA-1257604) and MAPK family signaling cascades (R-HSA-5683057) (Figures S7 and S8). The
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enrichment results highlighted that the most down-regulated DEGs, both in cardiomyocytes and coronary arterioles, are those encoding phosphoproteins (Figure S9).

Among these biological pathways and functional categories, several were enriched only by "exclusive DEGs," and more likely altered in a tissue-specific way. In fact, the KEGG and Reactome pathways analysis (Figure 3) on "exclusive DEGs" demonstrated an evident separation between the contributions of cardiomyocytes and arterioles in HCM and CTRLs, confirmed by corresponding GO and UniProt terms (Figures S11 and S12). Several pathways (Figure 3) related to the "translation process," such as "peptide chain elongation," "ribosome," "Nonsense Mediated Decay independent of/enhanced by the exon junction complex" were enriched by genes up-regulated in arterioles, while "regulation of actin cytoskeleton" and "focal adhesion" pathways, and pathways related to muscle contraction, such as "vascular smooth muscle contraction," or to signal transduction, such as "RHO GTPases activate PAKs/PKNs/ROCKs," were enriched by genes down-regulated in cardiomyocytes.

Notably, from the data stored in the IntAct database (Figure 4), among proteins with interactors enriched in genes exclusively down-regulated in cardiomyocytes, we found myosin IC (Myo1c), myosin XIX (MYO19), and myosin heavy chain 9 (MYH9); myosins are actin-based motor proteins that are required for multiple functions ranging from cytokinesis to muscle contraction.50

Among the down-regulated genes in arterioles, we found an enrichment of interactors for two member of SOX family, SRY-box transcription factor 2 and 15 (SOX2 and SOX15). The members of the SOX family of transcription factors widely expressed in development and participate in vasculogenesis and remodeling.51

4 | DISCUSSION

Hypertrophic cardiomyopathy is the most common genetic cardiomyopathy with a phenotype characterized by massive left ventricular hypertrophy (LVH), myocyte disarray, interstitial fibrosis, and...
coronary microvascular disease. The latter includes abnormal wall thickening of intramural coronary arterioles with lumen reduction which correlates with the decrease in maximum myocardial blood flow and coronary flow reserve.\textsuperscript{10}

Consistent evidence points to coronary microvascular dysfunction as a critical determinant of clinical progression and adverse outcome in HCM.\textsuperscript{14,16} However, little is known regarding the pathogenic mechanisms underlying this condition.

In recent years, whole transcriptome investigations by performing focused RNA-seq experiments and/or analyzing related datasets available in specialized online repositories revealed altered gene-expression profiles in HCM. Reported gene-enriched pathways involve immune modulation, signal transduction, hemostasis, metabolism, muscle contraction, inflammation, and fibrosis (TGF-β pathways).\textsuperscript{52–55}

Results published so far are based on the analysis of whole myocardial samples, including cardiomyocytes, vessels, fibroblasts, and interstitial tissue, and do not provide information on the possible existence of expression profiles selectively related to coronary microvascular dysfunction.

In the last decade, the combination of LCM and RNA-seq has been proven to be a useful tool to investigate cellular pathways underlying specific diseases and to identify potential therapeutic targets.\textsuperscript{56,57} Compared with other cell isolation techniques, LCM can precisely target and capture the cells of interest for a wide range of downstream assays.\textsuperscript{25,26,58,59}

To gain insight into the molecular mechanisms of CMD in HCM, we isolated remodeled arterioles and cardiomyocytes by LCM from frozen myectomy samples and investigated their respective transcriptome profiles by RNA-seq.
By applying this technique, we identified a total of 1485 differentially expressed genes (DEGs) in remodeled arterioles of HCM, of which 675 were over-expressed and 810 under-expressed. A lower number of DEGs (890) was detected in cardiomyocytes, of which 387 were up-regulated and 503 were down-regulated.

Interestingly, over 80% of the genes differentially expressed were exclusive of arterioles or cardiomyocytes ("exclusive DEGs" ie genes differentially expressed only in cardiomyocytes or in remodeled arterioles as compared to controls).

Pathway enrichment analysis of these "exclusive DEGs" recognized pathways specifically related to hypertrophic cardiomyocytes. We identified a down regulation of pathways correlated to muscle contraction, such as "vascular smooth muscle contraction" and "smooth muscle contraction" and signaling transduction, such as "RHO GTPases activate PAKs/PKNs/ROCKs." Rho GTPases are key regulators of different actomyosin-based cellular processes such as cell adhesion, cytokinesis, and contraction. The small GTP-binding proteins of the Ras family, such as RhoA, stabilize the actin cytoskeleton and promote the formation of focal adhesions.60–62 Accordingly, we also observed a down regulation of pathways related to "regulation of actin cytoskeleton" and "focal adhesion."

In contrast, remodeled arteries isolated by LCM showed selective alterations of pathways related to both the translation process (such as "peptide chain elongation," and "ribosome") and RNA quality control ("Nonsense Mediated Decay independent of/enhanced by the exon junction complex").

Interestingly, these pathways appear to be specific of remodeled arterioles in HCM since they have not been previously described in vascular remodeling associated with other cardiovascular diseases, including atherosclerosis and hypertension. Several studies, in fact, have highlighted the role of specific pathways related to immune/inflammatory process or to signaling transduction, such as Rho/ROCK pathways, Hippo/YAP signaling, and TGF-β pathway in vascular dysfunction associated with this condition.63–66

To the best of our knowledge, this is the first study that demonstrates the existence of distinctive pathways modifications between remodeled arterioles and cardiomyocytes in HCM patients and controls at the transcriptome level. The results obtained on isolated cardiomyocytes are overlapping, at least partially, with previous studies analyzing HCM myocardial homogenates.52–55 This might reflect the relative abundance of cardiomyocytes in the myocardial samples. Conversely, the association of LCM and RNA-seq allowed the detection of previously undescribed altered pathways, exclusive of remodeled arterioles and selective for HCM. This finding emphasizes the usefulness of this approach to analyze the molecular mechanisms underlying microvascular dysfunction and, possibly, to identify putative therapeutic targets.

4.1 Limitation of the study

The present study is a preliminary analysis, which requires validation by RT-PCR or Western blot analyses that have not yet been performed since all tissue samples collected for this study were used for NGS experiments. However, our main aim was to look for pathways specifically related to microvessel remodeling in HCM.

5 PERSPECTIVES

Our results highlight the usefulness of LCM/RNA-seq to identify specific molecular pathways related to CMD in HCM. Functional validation is needed to identify putative target genes amenable to future therapeutic approaches. To this purpose, we are collecting additional samples to extend our preliminary results and to perform a functional validation of the identified genes by RT-PCR and Western blot analysis.

AUTHOR CONTRIBUTIONS

AP, GdA, and PGC designed and supervised the study; AP, RC, and MGP performed the experiments; AP, LLP, FF, and GdA analyzed the data; AP, LLP, and GdA wrote the manuscript; LLP, FF, BC, and APe prepared figures; ML, DL, and IO collected clinical data; CF, IO, OER, and PGC critically revised the draft.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

We submitted the raw files (fastq) to the European Nucleotide Archive (ENA) under the project accession: PRJEB52994.

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