Identification of Hub Genes Associated with Immune Cells in Dilated Cardiomyopathy via Weighted Gene Co-expression Network Analysis

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Research

Keywords: Dilated cardiomyopathy, WGCNA, immune cells, bioinformatics, hub genes

Posted Date: July 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-652198/v1>

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Abstract

Background: Dilated cardiomyopathy (DCM) is the most common cardiomyopathy which account for a majority of heart failure. Although massive clinic experiments and gene profiling analyses on DCM have been conducted, the molecular mechanism of DCM associated with immune cells has not been fully elucidated. This study was designed to discover the immune mechanism of DCM using integrative bioinformatics analysis and provide new insights into the pathophysiology of DCM.

Methods: The GSE29819 dataset was downloaded, and Cibersort was applied to estimate the relative expression of 22 kinds of immune cells based on 14 samples of 7 DCM patients. Weighted gene co-expression network analysis (WGCNA) was performed to cluster the 2500 genes with the highest average expression into different modules and explore relationships between modules and immune cell types. Functional enrichment analysis was performed on key genes in significant modules identified by WGCNA and Cibersort. Key genes were then applied to Cytoscape to construct protein-protein interaction (PPI) network. Differentially expressed genes (DEGs) were identified based on DCM and normal controls in GSE29819 through R language. Hub genes were selected based on the DEGs and the genes identified by PPI and then verified via public GEO databases.

Results: The yellow and tan modules with 163 genes were identified as the key modules based on top 2500 DCM microarrays, significantly correlated with M1 and M2 macrophages. The intersection of newly screened 17 genes based on 163 key genes through Cytoscape and 2682 DEGs were defined as hub genes including CCT2, CCL2, and TXN. The results were finally verified via GSE116250 datasets.

Conclusions: The three hub genes associated with two immune cells identified by comprehensive bioinformatics analysis may play crucial roles in the pathophysiological mechanism of DCM, which provided potential immunological therapeutic targets and new insights into the treatment of DCM.

1. Introduction

Dilated cardiomyopathy (DCM) is an inheritable disease with structural or functional abnormalities of the myocardium and a crucial reason of heart failure and sudden cardiac death [1]. Recent studies have concentrated on the genetic and pathogenic mechanisms of DCM associated with immune system [2]. Increasing evidence indicated that gene expression patterns and immune infiltrate were compatible with immune cell activation from DCM patients [1; 3; 4; 5]. Immune cells release cytokines to promote remodeling, collagen deposition and fibrosis [6; 7]. Nevertheless, immune modular analysis of gene expression profiles for DCM still needs further research to clarify the cardiac immunopathology as they affect the treatment of DCM.

Weighted Gene Co-expression Network Analysis (WGCNA), a comprehensive biology method of R functions to cluster genes into modules based on the expression patterns of different samples. According to the interconnectivity of genes, the relationships between the modules and the phenotypes to explore the therapeutic targets can be obtained by WGCNA [8]. It has been applied to massive biological process analyses [9; 10]. Along with Cell type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) [11; 12], a versatile computational method with high resolving power for quantifying the relative immune cell fractions from ~ 500 marker gene expressions profiles, the key genes associated with immune cells from co-expression significant modules in DCM dataset were screened. We then conducted the functional enrichment analysis and constructed protein-protein interaction (PPI) of based on the genes. Hub genes were screened according to the PPI network and differentially expressed genes (DEGs). We anticipated that this study could provide potential targets for the treatment of DCM.

2. Methods And Materials

2.1 Data Sources and Processing

14 gene microarray expression profiles from left (LV) and right ventricular (RV) myocardial samples of DCM patients and 12 expression profiles from LV and RV myocardial samples of non-failing (NF) donor hearts, were downloaded from the GSE29819 dataset in Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). The original expression profile of the GSE29819 dataset was processed including gene ID translation and data filter by R language.

2.2 Identification of immune cell composition

CIBERSORT, a bioinformatics algorithm, was applied to estimate the 22 immune cell composition based on the gene expression profiles. In our study, the fractions of immune cell subpopulations in the 14 DCM samples of GSE116250 were estimated by CIBERSORT (<https://cibersort.stanford.edu/>). The gene expression matrix data were quantile normalized before and the permutations were set at 100.

2.3 WGCNA analysis

The WGCNA [8] by R language (<https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/>) was applied to construct the gene co-expression network based on 2500 highest expression genes of DCM patients. The scale-free topology fit index was 0.9, the minimum number of genes in each module was 20, the threshold for module merging was 0.25, and the sensitivity was 2. Then genes were clustered into different modules with different colors through the average linkage hierarchical clustering method. Pearson correlation method was utilized to calculate the correlation between modules and the expression of immune cells was estimated by Pearson correlation method. The modules with the correlation coefficient > 0.7 were defined as significant modules.

2.4 Functional Enrichment Analyses

The genes in significant modules were used to conduct functional enrichment analysis. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed by the R package 'clusterProfiler' (<http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html>) [13; 14]. The enrichment results of GO revealed the biological process (BP), molecular function (MF), cellular component (CC), and pathways related to key genes in the interested modules. P -value < 0.05 was set as the significant enrichment, and of the results that exceeded the threshold, the top 4 terms of GO domain and top 4 KEGG pathways were identified.

2.5 PPI network construction

The key genes selected above were imported into the online Search Tool for the Retrieval of Interacting Genes (STRING, <https://string-db.org/>) [15] to generate the PPI network. The minimum required interaction score was set at 0.4 to identify multiple protein interactions. Nodes represent proteins, edges indicate both functional and physical protein correlations in the PPI network, and line colors indicate the type of interaction evidence.

2.6 Identification of DEGs

The Linear Models for Microarray data (limma) package [16] by R language (<https://bioconductor.org/packages/release/bioc/html/limma.html>), was utilized to elucidate the DEGs between NF control hearts and those suffering from DCM. Genes with adjusted P -value < 0.05 and $|\text{Log}_2 \text{ Fold change (Fc)}| > 1$ were considered as DEGs. The ggplot2 package [17] by R language

(<https://www.bioconductor.org/packages/release/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html>) was used to generate the volcano plot.

2.7 Identification and validation of hub genes

The results from the STRING database were applied to CytoHubba [18], a plug-in of the Cytoscape software (v3.8.2, <http://manual.cytoscape.org/en/stable/Styles.html>) to screen hub genes. The 3 algorithms in CytoHubba including Degree, EPC, and BottleNeck were utilized to obtain the union set of the top 10 genes generated in each algorithm. The intersection of the union genes and DEGs were differentially expressed immune-related genes and were regarded as hub genes. GSE116250, the public dataset with the largest number of samples of RNA-seq for DCM, was used for validation of hub genes.

3. Results

The analysis process of the present study to screen the key modules, hub genes, and immune cells involved in the immunological pathogenesis of DCM is shown in Fig. 1.

3.1 Immune cell infiltration analysis

The immune cell infiltration analysis was conducted to explore the potential immune mechanisms. 22 differential immune cell subpopulations in the CIBERSORT algorithm based on gene microarrays of DCM patients from GSE29819 were shown in Fig. 2.

3.2 Co-expression modules construction

WGCNA analysis was utilized to conduct the Pearson correlation test based on the immune cell types. The cluster analysis results of the DCM samples were shown in Fig. 3A. A soft-threshold power, which affected the scale independence and mean connectivity of the network, was introduced into the network topology. A soft-threshold of 9 with a scale independence of 0.9 was utilized to obtain the approximate scale-free topology (Fig. 3B). As shown in Fig. 3C, 29 modules with co-expression traits were screened.

3.3 Correlation between modules and immune cells

Correlation analysis was carried out between modules and 22 kinds of immune cells. Key modules were extracted with the correlation coefficient > 0.7 . As shown in Fig. 4, the yellow module ($r = 0.86$) was the most significantly positively correlated with M1 macrophages, and the tan module showed the second strongest positive correlation with M2 macrophages ($r = 0.76$). These two modules with 163 genes were identified as the significant modules of interest.

3.4 Function enrichment analyses

The 163 genes mentioned above were used to evaluate the biological function via GO and KEGG pathway enrichment analyses. The GO analysis results showed that they were significantly enriched in response to interferon-gamma, response to oxidative stress, antigen processing, and presentation of exogenous peptide antigen for the BP category (Fig. 5A). For the MF category, the genes were enriched in peptide antigen binding, amide binding, antigen binding, and G protein-coupled receptor binding (Fig. 5B). In addition, the CC category analysis showed they were significantly enriched in ER to Golgi transport vesicle membrane, COPII-coated ER to Golgi transport vesicle, Golgi-associated vesicle membrane, and Golgi-associated vesicle (Fig. 5C). The KEGG pathway enrichment analysis results showed that

they were enriched in pathways including infectious disease: bacterial, immune system, metabolism, and transport and catabolism (Fig. 5D).

3.5 DEGs analysis

The DEGs were screened from the gene profiles of GSE29819 including 26 samples of paired left and right ventricular myocardial tissue from 7 DCM patients and 6 normal controls, in order to get the gene signature of DCM associated with immune cells. By principal component analysis (PCA), the DEGs demonstrated significant individual differences and distinct intergroup bias (Fig. 6A). A total of 2,682 genes, including 1318 upregulated DEGs and 1364 downregulated DEGs (Fig. 6B) were identified. Top 50 DEGs were shown in the heatmap (Fig. 6C).

3.6 Identification and validation of hub genes

Finally, we conducted PPI network construction of the 163 key genes. As shown in Fig. 7, a total of 164 nodes and 299 edges were identified from the PPI network. 3 algorithms of the CytoHubba were then applied to identify the top 10 genes, respectively, based on the PPI network, as shown in Fig. 8A-C. The union set of these genes (a total of 17 genes) were used for further hub genes identification. In detail, Venn diagram Fig. 8D showed the intersection between the identified genes by 3 algorithms and the DEGs, including containing TCP1 subunit 2 (CCT2), C-C motif ligand 2 (CCL2), and thioredoxin (TXN), which were determined as hub genes. The GEO dataset GSE116250 was used to estimate whether the levels of these three genes between DCM and NF were consistent with our results. The gene expression levels were normalized by the fragments per kilobase of transcript per million mapped reads (FPKM). The FPKM values of CCT2, CCL2, and TXN genes were extracted, and the extreme values were filtered out. As shown in Fig. 8E, the expression levels of CCL2 were significantly higher, while the expression levels of CCT2 and TXN were significantly lower in DCM than those in NF. In conclusion, we revealed that CCT2, CCL2, and TXN may play important roles in the molecular mechanisms and immune microenvironment underlying DCM.

4. Discussion

Recently, few systematic studies have been performed to elucidate the precise role of immune infiltration in DCM. Thus, we explored the hub genes and immune cells that were differently expressed between DCM and NF, which may provide potential therapeutic targets. In the present study, we identified M1, M2 macrophages, and 163 key genes associated with immune cells from DCM patients through WGCNA network analysis and CIBERSROT algorithm. To the best of our knowledge, we are the first to explore the immune etiological mechanisms of DCM by WGCNA. These key genes were then applied to function enrichment analysis. According to GO analysis, key genes were most enriched in response to interferon-gamma (BP), peptide antigen binding (MF), and ER to Golgi transport vesicle membrane (CC). KEGG pathway analysis indicated that key genes were mostly enriched in infectious disease. Meanwhile, we found three macrophage-correlated regulators (CCT2, CCL2, and TXN) that were significantly associated with DCM patients based on the PPI network and CytoHubba. The expression levels of these three genes were also verified in the public database.

In the present study, M1 macrophages in the yellow module and M2 macrophages in the tan modules were extracted as the most and second correlated immune cells with DCM. Consistent with us, previous studies have demonstrated that macrophages promoted deterioration of the failing myocardium that occur in DCM [19; 20], thus leading to dysfunction. The M1 macrophages are known as the proinflammatory phenotype that secretes proinflammatory cytokines [21]. Several studies [22; 23] have found that M1 macrophages among the total cells were predominant in the DCM hearts. The M2 phenotype is characterized by producing anti-inflammatory response, and inducing collagenogenesis [24]. A recent study [25] has also found that phenotypic polarization toward M2 macrophages may be associated with collagen formation and then ventricular remodeling in DCM. These results seemed to be

paradoxical, we speculated M1 and M2 macrophages may play different roles in DCM where myocardium damage and fibrosis develop simultaneously.

In the present study, CCT2, a member of the chaperonin containing TCP1 complex, was found in the tan module associated with M2 macrophages and may serve as one of the predictors of DCM patients. Nowadays, researches have been performed to investigate the relationship between CCT2 and immune system. For instance, Li et al. [26] explored CCT subunit genes expression and immune cell infiltration using TIMER database, they found that CCT subunit gene including CCT2 showed a strong correlation with macrophages, which were consistent with our study that macrophages were screened as the significantly correlated immune cell with DCM. Zhang et al. [27] found that the viability of wild-type macrophages was much higher than CCT1-deficient macrophages where CCT2 was significantly induced, indicating increased CCT2 may have a negative correlation with macrophages. Another study [28] revealed that inhibiting CCT2 altered the levels of other CCT subunits, which induced immunogenic cell death to stimulate a robust anti-cancer immune response. These studies elucidated that CCT2 may provide an immunosuppressive effect in various diseases, thus providing a therapeutical effect. In the field of heart disease, reduced CCT2 gene expression has been previously reported in failing hearts than NF hearts for end-stage ischemic heart failure [29]. However, little is known about the expression levels of CCT2 in DCM. We suggested that CCT2 expression level was negatively related with DCM, which needs further in vivo and in vitro studies to clarify the role of CCT2 in the pathogenesis of DCM.

In our study, CCL2 in yellow module associated with M1 macrophage was screened as hub gene in DCM. CCL2 is known for driving the chemotaxis of myeloid cells including macrophages to impact their polarization, adhesion, and effector molecule secretion in inflammatory conditions [30]. For instance, CCL2 has been suggested to impact on the polarization of both M1 and M2 macrophages, which seems to be context dependent [31; 32; 33]. In DCM, the presence of CCL2 in endomyocardial biopsy tissue has been reported to contribute to enhanced inflammatory macrophages (M1) infiltration and activation, thus resulting in chronic damage of cardiomyocytes [34] in the early days. However, recent researchers have suggested that CCL2 is one of the four stem cell chemo-attractant cytokines to have a benefit effect on tissue repair [30; 35]. CCL2 could partly be responsible for the myocardial homing factor of mesenchymal stem cells (MSCs) [36], which promoted MSCs migration to the dilated heart and supported the functional recovery of DCM [37]. These paradoxical findings may attribute to the complexity of the effects of CCL2 on immune cell behavior that the interaction of CCL2 with their receptor has a particular role within a specific physiological/pathological context. Thus, a better understanding of how CCL2 interacts with immune cells would be pivotal to the improvement of existing therapeutic approaches for DCM. It led us to hypothesize that CCL2 may promote DCM initialization and development at early stage of DCM, while promote recovery of DCM patients with impaired cardiac function. In this way, targeting CCL2 may serve as the therapeutic options of DCM patients at different stages, which still needs further studies to clarify.

In our bioinformatic analysis, TXN in tan module associated with M2 macrophages was screened, which may play a protective role in DCM pathophysiology. TXN encodes the protein thioredoxin-1 (TRX-1), a class of ubiquitous oxidoreductase with strong co-cytokine, chemoattractant, is involved in many redox reactions[38; 39]. Although the knowledge of TRX-1 immune targets is now expanding, little is known about how TRX-1 selects the substrates and how TRX-1 is involved in different redox-mediated immune responses. There have been studies exploring the correlation of TRX-1 and immune cells especially macrophages. For example, Hadri et al. [40] revealed that TRX-1 promoting macrophage polarization into the M2 phenotype could explain its protective effects in atherosclerosis. This anti-inflammation may be through ameliorating oxidative stress for the causal link between these two factors [41]. These data provided novel insight into the relationship between oxidative stress and cardiovascular diseases. TRX family have also been increasingly reported to explore the TRX levels in serum and in myocardial tissue of DCM patients. For example, Kishimoto et al. [42] have found that the serum TRX-1 levels were higher in DCM patients than

normal controls. In humans, another member of TRX family, TRX-2, encoded by TXN-2, is a crucial mitochondrial protein that suggested to regulate redox signaling in DCM filed. Huang et al. [43] found that TRX-2 protein expression levels were reduced in hearts from DCM patients. Moreover, they suggested that mice with cardiac-specific TRX-2 deletion developed spontaneous DCM. However, as we know, few studies have explored the role of TRX-1 in the cardiac tissues of DCM patients. We speculated that TRX-1 would ameliorate cardiac function of DCM patients through suppressing reactive oxygen species production, and then promoting anti-inflammatory responses with polarizing M2 phenotypes.

Conclusions

In this study, we conducted immune infiltration and WGCNA analysis to propose the regulatory mechanisms of DCM for the first time. Three hub genes (CCT2, CCL2 and TXN) associated with two kinds of immune cells (M1 and M2 macrophages) identified in our study may be pivotal in DCM. These novel genes with correlated macrophages are worthy of further mechanistic studies on more patients to support our results and to provide potential therapeutic strategies for DCM patients.

Abbreviations

BP: biological process

CC: cellular component

CCL2: C-C motif ligand 2

CCT2: TCP1 subunit 2

CIBERSORT: Cell type Identification By Estimating Relative Subsets of RNA Transcripts

DCM: dilated cardiomyopathy

DEGs: differentially expressed genes

FC: fold change

FPKM: fragments per kilobase of transcript per million mapped reads

GEO: Gene Expression Omnibus

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

Limma: Linear Models for Microarray data

LV: left ventricular

MF: molecular function

MSCs: mesenchymal stem cells

NF: non-failing

PCA: principal component analysis

PPI: protein-protein interaction

RV: right ventricular

STRING: Search Tool for the Retrieval of Interacting Genes

TXN: thioredoxin

TRX-1: thioredoxin-1

WGCNA: weighted gene co-expression network analysis

Declarations

Ethics approval and consent to participate:

We acknowledge GEO database for providing their platforms and contributors for uploading their meaningful datasets.

Consent for publication:

Not applicable.

Availability of supporting data:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests:

The authors declare that they have no competing interests.

Funding:

This work was supported by grants from National Nature Science Foundation of China (No. 81530011).

Authors' contributions:

Jielin Deng analyzed the patient data regarding DCM. Jielin Deng and Yunqiu Jiang were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements:

Not applicable.

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Figures

Figure 1

Flow chart of analysis process in the present study. WGCNA algorithm and CIBERSORT were utilized to perform the gene co-expression module construction analysis. The GO and KEGG analysis were conducted based on key genes in significant modules to explore the onset of DCM. Hub genes that may be involved in the DCM development were identified through the PPI network and DEGs. WGCNA: Weighted Gene Co-expression Network Analysis; CIBERSORT: Cell type Identification By Estimating Relative Subsets Of RNA Transcripts; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DCM: dilated cardiomyopathy; PPI: protein-protein interaction; DEGs: differentially expressed genes.

Input Sample	B cells naive	B cells memory	Plasma cells	T cells CD8 naive	T cells CD4 memory resting	T cells CD4 memory activated	T cells CD4 memory follicular helper	T cells regulatory (Tregs)	T cells gamma delta	NK cells resting	NK cells activated	Monocytes	Macrophages M0	Macrophages M1	Macrophages M2	Dendritic cells resting	Dendritic cells activated	Mast cells resting	Mast cells activated	Eosinophils	Neutrophils
GSM739002	0	0.092	0.081	0	0	0.161	0.012	0	0.08	0	0	0.12	0	0.026	0.209	0	0	0.139	0	0	0.08
GSM739003	0	0.052	0.108	0	0	0.176	0	0.009	0.005	0	0	0.086	0.131	0.011	0.01	0.178	0	0	0.239	0	0
GSM739004	0	0.04	0.031	0.055	0.037	0.16	0	0.073	0	0.014	0.007	0.063	0	0.025	0.005	0	0	0.123	0	0	0.01
GSM739005	0	0.159	0.073	0.023	0	0.204	0	0	0	0	0	0.022	0	0	0.008	0.005	0.005	0.159	0	0.042	0
GSM739006	0.044	0.015	0.04	0	0.072	0.191	0	0	0	0	0.041	0.05	0	0	0.005	0	0.006	0.167	0	0	0.023
GSM739007	0	0.206	0.059	0	0	0.189	0	0	0.019	0	0.038	0	0.05	0	0	0.204	0	0.002	0.166	0.003	0.063
GSM739008	0	0.02	0.022	0.07	0	0.202	0	0	0.028	0	0.009	0.144	0	0.05	0.005	0	0	0.11	0	0	0.024
GSM739009	0	0.011	0.049	0.031	0	0.199	0	0	0.027	0	0.019	0.109	0.015	0.089	0.008	0.005	0	0.095	0	0	0.007
GSM739010	0	0.046	0.027	0	0.027	0.163	0	0.09	0	0	0.028	0.058	0	0	0.008	0.007	0.001	0.205	0	0.016	0
GSM739011	0	0.19	0.057	0	0	0.068	0	0.067	0.019	0	0.034	0.036	0.032	0.015	0.257	0	0	0.2	0	0	0.025
GSM739012	0.04	0	0.072	0.031	0	0.247	0	0.064	0	0	0.077	0.081	0	0.007	0.172	0	0	0.2	0	0.008	0
GSM739013	0	0.246	0.093	0	0.008	0.239	0	0.046	0	0	0.067	0	0	0	0.058	0.089	0.001	0.152	0	0	0
GSM739014	0.051	0	0.007	0	0	0.151	0	0.023	0.012	0	0.029	0.087	0	0	0.005	0	0	0.146	0	0	0
GSM739015	0.094	0	0.038	0.019	0	0.161	0	0.136	0	0.037	0	0.107	0	0	0.261	0	0	0.147	0	0	0

Figure 2

The expression of 22 immune cell subtypes in 14 samples of DCM patients estimated using CIBERSORT, which was higher from blue to red. DCM: dilated cardiomyopathy; CIBERSORT: Cell type Identification By Estimating Relative Subsets Of RNA Transcripts

Figure 3

WGCNA analysis. (A) A total of top 2500 expressed genes of DCM samples were clarified into two clusters. (B) The network topology including the scale independence and the mean connectivity of gene co-expression modules for DCM. (C) Dendrogram of gene co-expression modules of DCM. WGCNA: Weighted Gene Co-expression Network Analysis; DCM: dilated cardiomyopathy.

Figure 4

Module trait relationships of 22 immune cells and modules revealed by heatmap. The coefficient was higher from blue to red.

Figure 6

DEGs analysis. (A) 1318 upregulated (red) and 1364 downregulated DEGs (blue) were identified between DCM patients and NF donors in GSE29819. (B) PCA between DCM (green) and NF (blue). (C) Top 50 DEGs shown in heatmap. DEGs: differentially expressed genes; DCM: dilated cardiomyopathy, NF: non-failing; PCA: principal component analysis.

Figure 7

PPI network construction based on 163 key genes using the STRING database. PPI: protein-protein interaction; STRING: Search Tool for the Retrieval of Interacting Genes.

Figure 8

Identification and validation of hub genes. Three algorithms including (A) Degree (B) EPC, and (C) Bottleneck obtained from the PPI network. (D) Venn diagram showed the intersection (red lines) of genes screened from PPI network and DEGs. (E) RPKM values of CCT2, CCL2 and TXN in DCM patients and NF donors in GSE116250. * $P < 0.05$ DCM vs. NF. PPI: protein-protein interaction; DEGs: differentially expressed genes. RPKM: Reads Per Kilobase of transcript perMillion mapped reads; CCT2: containing TCP1 subunit 2; CCL2: C-C motif ligand 2; TXN: thioredoxin; DCM: dilated cardiomyopathy, NF: non-failing.