Disease Markers

Biomarkers in Heart Failure and Associated Diseases

Lead Guest Editor: Andrea Salzano
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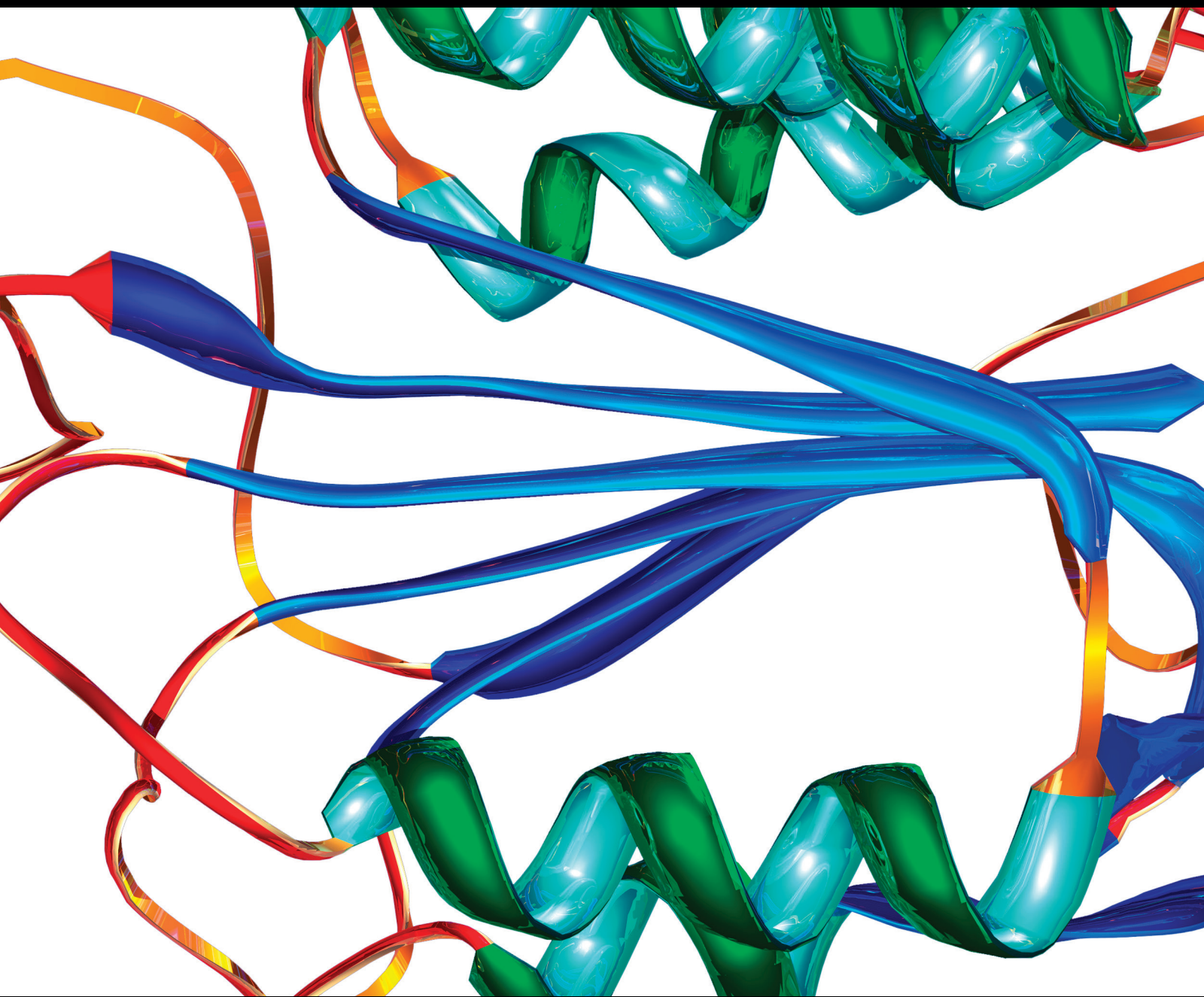

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Editorial Board

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Editorial

Biomarkers in Heart Failure and Associated Diseases

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Despite considerable improvement in the management of heart failure (HF), unsustainable levels of morbidity and mortality coupled with an increasing economic and social burden have been observed over the previous three decades [1]. A rational explanation of this is the fact that no single pathophysiologic paradigm of HF has been clarified, resulting in failure of our current models to completely explain disease progression [2].

Classically, seven categories of biomarkers in HF have been described, reflecting the different pathophysiological pathways involved in disease progression [3]. These include myocardial stretch, myocyte injury, matrix remodelling, inflammation, neurohumoral activation, oxidative stress, and indices of renal dysfunction [4]. Moreover, growing evidence supports the key role of alternative pathophysiological pathways (e.g., the gastrointestinal system, the anabolic/catabolic imbalance, and multiple hormonal deficiency syndrome), with ever-increasing identifications of novel biomarkers that demonstrate their importance in HF [5–9].

In this context, a growing interest in multimarker approaches to biomarker panels to assess multiple pathophysiologic pathways has been realised, including the combined use of proteins, lipids, metabolites, hormones, and genetic markers [10].

Owing to these recent advances in biomarker research, the aim of this special issue was to focus on the role of biomarkers in HF and associated diseases.

Ischemic heart disease is to date the most frequent cause of HF [2], with atherosclerosis the major pathophysiological mechanism. In this issue, L.-D. Mocan Hognogi et al. reviewed the role of adipokines (in particular visfatin, apelin, leptin, and resistin) as biomarkers of ischemic cardiac disease and concluded that "*there is no doubt that inflammation is viewed as an important pathophysiological step in the development of atherosclerosis*."

Importantly, the identification of patients at high risk of poor prognosis is one of the principal aims of current clinical research [11]. With this regard, M. Alavi-Moghaddam et al. conducted a pilot study involving 21 patients diagnosed with acute myocardial infarction and demonstrated that plasma levels of microRNA-208b, of which levels of expression have been demonstrated to be increased in the blood of patients with acute myocardial infarction, were 2-fold higher in patients who died after 6 months than in those which survived.

Further, J. Banach et al. investigated plasma concentration of procalcitonin (PCT) in 130 patients with chronic HF with reduced ejection fraction, assessing its prognostic value during a 24-month follow-up period. Indeed, PCT levels were significantly higher in HF patients when compared to a control group. Further, Kaplan-Meier survival curves revealed that patients with PCT in the highest quartile had a significantly reduced probability of survival. This is additional evidence supporting the role of inflammation in HF [7].

Diabetic cardiomyopathy (DCM) is a common cardiac dysfunction, affecting approximately 12% of diabetic patients, and is featured by ventricular diastolic and (or) systolic dysfunction. N. Li et al. provided a comprehensive and novel illustration of gene expression profiles to identify differentially expressed genes in myocardial tissue, which may play critical roles in the occurrence and development in patients with DCM. This is of great interest considering that diabetes mellitus has been described in approximately 20-25% of HF patients [5, 12].

HF is a progressive condition in which myocardial damage, caused by cardiovascular risk factors, leads to the development of myocardial dysfunction. Thus, an ever-worsening condition is present until the patient eventually develops end-stage heart failure. Heart transplantation is the only survival option for end-stage patients [2]. Cardiac allograft vasculopathy (CAV) is the leading cause of cardiovascular adverse events during follow-up of heart transplantation. S. Mirabet et al. demonstrated that high-sensitivity cardiac troponin T, measured during a long-term follow-up, appears as a helpful biomarker to identify patients at low risk of adverse CV outcomes. On the other hand, the soluble form of AXL (sAXL) and a biomarker of endothelial dysfunction was not able to predict outcome.

In conclusion, this issue collected novel findings and shed light upon the role of biomarkers in HF.

Conflicts of Interest

Other authors declare that they have no conflicts of interest.

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Marco Proietti
Valeria Raparelli
Liam M. Heaney

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Research Article

Identification of Core Gene Biomarkers in Patients with Diabetic Cardiomyopathy

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Guest Editor: Liam M. Heaney

Diabetic cardiomyopathy (DCM) is a disorder of the myocardium in diabetic patients, which is one of the critical complications of diabetes giving rise to an increased mortality. However, the underlying mechanisms of DCM remain incompletely understood presently. This study was designed to screen the potential molecules and pathways implicated with DCM. GSE26887 involving 5 control individuals and 7 DCM patients was selected from the GEO database to identify the differentially expressed genes (DEGs). DAVID was applied to perform gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. A protein-protein interaction (PPI) network was also constructed to visualize the interactions among these DEGs. To further validate significant genes and pathways, quantitative real-time PCR (qPCR) and Western blot were performed. A total of 236 DEGs were captured, including 134 upregulated and 102 downregulated genes. GO, KEGG, and the PPI network disclosed that inflammation, immune disorders, metabolic disturbance, and mitochondrial dysfunction were significantly enriched in the development of DCM. Notably, IL6 was an upregulated hub gene with the highest connectivity degree, suggesting that it may interact with a great many molecules and pathways. Meanwhile, SOCS3 was also one of the top 15 hub genes in the PPI network. Herein, we detected the protein level of STAT3 and SOCS3 in a mouse model with DCM. Western blot results showed that the protein level of SOCS3 was significantly lower while phosphorylated-STAT3 (P-STAT3) was activated in mice with DCM. *In vitro* results also uncovered the similar alterations of SOCS3 and P-STAT3 in cardiomyocytes and cardiac fibroblasts induced by high glucose (HG). However, overexpression of SOCS3 could significantly reverse HG-induced cardiomyocyte hypertrophy and collagen synthesis of cardiac fibroblasts. Taken together, our analysis unveiled potential biomarkers and molecular mechanisms in DCM, which could be helpful to the diagnosis and treatment of DCM.

1. Introduction

Diabetic cardiomyopathy (DCM) is a common cardiac dysfunction which affects approximately 12% of diabetic patients, giving rise to overtly higher cardiovascular morbidity and mortality than those without glycemia [1]. DCM is featured by ventricular diastolic and (or) systolic dysfunction occurring in patients with type 1 or type 2 diabetes independent of hypertension, coronary artery disease (CAD), and other cardiovascular diseases [2]. The pathogenesis of DCM is a multistep process, which is implicated with the alterations of various vital events, including mitochondrial dysfunction, altered lipid metabolism, endoplasmic reticulum stress, oxidative stress, inflammation, and epigenetic changes [3, 4]. Evidence is mounting that the occurrence and progression of DCM are triggered by the abnormal expression or mutation of genes, such as S6 kinase 1 (S6K1) [5], CD36 [6], peroxisome proliferator-activated receptor-α (PPAR-α) [7], and protein kinase C (PKC) [8]. Currently, the diagnosis of DCM in clinics mainly relies on the serum natriuretic peptide (NAPP) level and other noninvasive tests involving electrocardiography, to clarify ventricular overload; X-ray, to evaluate fluid accumulation; and conventional cardiac ultrasound, to figure out cardiac structure and function. However, these methods for DCM diagnosis lack the specificity and efficiency; it was thus difficult for us to obtain early and

accurate diagnosis as well as treatment, so some patients with DCM missed the best opportunity for treatment, thereby increasing death risk [1]. Hence, identifying the specific and sensitive genes or proteins which can help us confirm the patients with DCM as early as possible is of vital significance, not only for more accurate diagnosis, better treatment, and ideal prognosis but also for an overall understanding of the molecular mechanisms underlying DCM.

Bioinformatic analysis and gene expression profiling analysis have enabled us to screen molecular markers among healthy individuals and patients, which provides novel insights into diseases at multiple levels ranging from the alterations of copy number at the genome level to gene expression at transcriptome level, and even epigenetic alterations. However, in fact, the application of these microarrays has not gained popularity as expected in clinics because of an overwhelming amount of genes identified by gene profiling, lack of validation or repeatability, and intricate statistical analyses [9–11]. Therefore, for the purpose of putting these expression profiles in clinical practice as quickly as possible, it is of necessity to validate a suitable amount of genes and develop a proper and ideal approach which could be operated routinely.

In the current study, the gene expression profile of GSE26887 was downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and analyzed using the GEO2R online tool to comprehensively identify the differentially expressed genes (DEGs) between DCM and healthy individuals. Furthermore, we also analyzed the gene ontology (GO) involving biological process (BP), molecular function (MF), cellular component (CC), and KEGG pathways of these DEGs. Subsequently, we carried out a protein-protein interaction (PPI) network of these DEGs and chose the top 15 hub genes with a high degree of connectivity. Meanwhile, we also re-identified the top 15 hub genes by PCR and Western blot.

2. Materials and Methods

2.1. Microarray Data. We obtained the microarray of GSE26887 from the National Center for Biotechnology Information (NCBI) GEO database, which is a free and publicly available database [10]. The GSE26887 dataset possesses 24 samples in all, containing 5 normal individuals, 7 patients with diabetic cardiomyopathy, and 12 nondiabetic-heart failure patients with ischemic cardiomyopathy, which was based on the GPL6244 platform [HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array [transcript (gene) version] by Greco et al. We also downloaded the Series Matrix File of GSE26887 from the GEO database in Pubmed. All of these cardiac tissues were acquired from the vital, noninfarcted zone derived from patients with dilated hypokinetic postischemic cardiomyopathy in surgical ventricular restoration. Inclusion criteria of the diabetic group for this microarray were blood glucose ≥126 mg/dL, previous type 2 diabetes mellitus (T2DM) diagnosis, or receiving antidiabetic therapy, and those for the nondiabetic group were blood glucose <100 mg/dL and HbA1c n.v. 4.8–6.0%. Additionally, heart failure patients were matched for ejection fraction (LVEF), end systolic volume (ESV), sex, age, smoke habits, ethnic distribution, body mass index (BMI), hypertension, and glomerular filtration rate. The gene expression profile was assessed by Affymetrix GeneChip Human Gene 1.0 ST array using total RNA extracted from the above samples.

2.2. Identification of DEGs. We screened the DEGs between DCM and healthy samples using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r), an interactive analysis tool for the GEO database on the basis of R language. Consistent with the previous criteria [12, 13], we defined the genes with log FC < −1 (downregulated genes) or logFC > 1 (upregulated genes) as differentially expressed. Meanwhile, the adjusted P value < 0.05 was regarded statistically different, aiming at reducing the false positive rate. Furthermore, after downloading the relatively raw TXT data, we also applied visual hierarchical cluster analysis to display the volcano plot and heat map of DCM and healthy samples using ImageGP (http://www.ehbio.com/ImageGP/index.php/Home/Index/index.html).

2.3. Protein-Protein Interaction (PPI) Network. The PPI network could identify the core hub genes and key gene modules between healthy individuals and patients [14]. Firstly, we used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), which is a well-known database containing the predicted and recognized protein interactions (https://string-db.org/), to identify the PPI association. Subsequently, we applied Cytoscape software platform on the basis of the PPI associations to construct the PPI network. Top 15 hub genes were selected according to the ranking order of connectivity degree.

2.4. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis. GO analysis can annotate a collection of genes with functions involving molecular function (MF), cellular components (CC), and biological process (BP) [15]. The KEGG pathway is a group of databases which could hint biological pathways of certain genes implicated with diseases and drugs. KEGG in essence is a resource for us to receive an integrated understanding of biological functions and even some advanced genome information [16]. The GO and KEEG analysis in our study was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.ncifcrf.gov) (version 6.7), an online biological function database integrating considerable biological data and analysis tools [17]. $P < 0.05$ should be the cut-off criterion. We also used ImageGP to construct the enrichment plots, aiming to visualize the BP, MF, CC, and KEGG pathways of these DEGs.

2.5. Animals. All animal experimental procedures in this study were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University and were performed in accordance with the Care and Use of Laboratory Animals published by the US National Institute of Health (Revised 2011). Both male type 2 diabetic (db/db) ($n = 8$) and WT mice ($n = 8$) (8–10 weeks) weighing 25.2 ± 2 g were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China).

2.6. Quantitative Real-Time PCR (qPCR). The mice were sacrificed by injecting excessive sodium pentobarbital. Whereafter, the left ventricles of mice were collected for further RNA detection. Total RNA was isolated using the TRIzol (Invitrogen, Carlsbad, CA, USA) assay, the concentrations and purities of which were quantified using an ultraviolet spectrophotometer. The RNA was then reversely transcribed according to the previous description [18]. The expression levels of top 5 upregulated genes and top 5 downregulated genes were normalized to GAPDH. Relative mRNA expression levels were analyzed by the $2^{-\Delta\Delta}$ cycle threshold (CT) method. The primer sequences are displayed in Table S1.

2.7. Western Blot. Protein extraction, SDS-PAGE, and immunodetection of the cardiac tissues were all performed according to our previous research. Protein expression levels were normalized to the matched total proteins or GAPDH [19].

2.8. Cell Culture and Treatment. Neonatal rat cardiomyocytes and neonatal rat cardiac fibroblasts were isolated according to the previous study [20]. Cardiomyocyte hypertrophy was evaluated by anti-α-actinin immunofluorescence staining while the phenotypic change of cardiac fibroblasts was evaluated by anti-α-SMA immunofluorescence staining. For cell transfection, replication-defective adenoviral vectors were employed to upregulate the expression of SOCS3. After infection, cardiomyocytes and cardiac fibroblasts were incubated with a high-glucose concentration (33 mM glucose) while the normal group was exposed to a normal glucose concentration (5.5 mM glucose).

2.9. Statistical Analysis. The obtained data were presented as mean ± SD (standard deviation) and assessed by the two-tailed Student's t-test. A difference of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of DEGs. The overall flow diagram of our study is presented in Figure 1. In this study, a total of 5 normal individuals and 7 patients with DCM were analyzed. We applied the GEO2R online analysis tool with default parameters to screen the DEGs, using adjusted P value < 0.05 and logFC ≤ −1 or logFC ≥ 1 as the cut-off criteria. After analyzing GSE26887, 236 DEGs were captured, including 134 upregulated genes and 102 downregulated genes. The expression proportion of these DEGs is displayed in the volcano plot (Figure 2(a)). The heat map represented the top 25 upregulated genes and top 25 downregulated genes between patients with DCM and healthy individuals (Figure 2(b)). Among these 236 DEGs, the top 5 upregulated genes involved NPPA, SFRP4, DSC1, NEB, and FRZB while the top 5 downregulated genes were SERPINE1, SERPINA3, ANKRD2, XRCC4, and S100A8. The gene tiles and biological functions of these 10 genes are displayed in Table 1.

To ensure the credibility of the microarray of GSE26887 and obtain further credible analysis, we re-identified the top 5 upregulated genes and top 5 downregulated genes via qPCR in vivo and in vitro. The results of echocardiography, hematoxylin and eosin (H&E) staining, and picrosirius red (PSR)

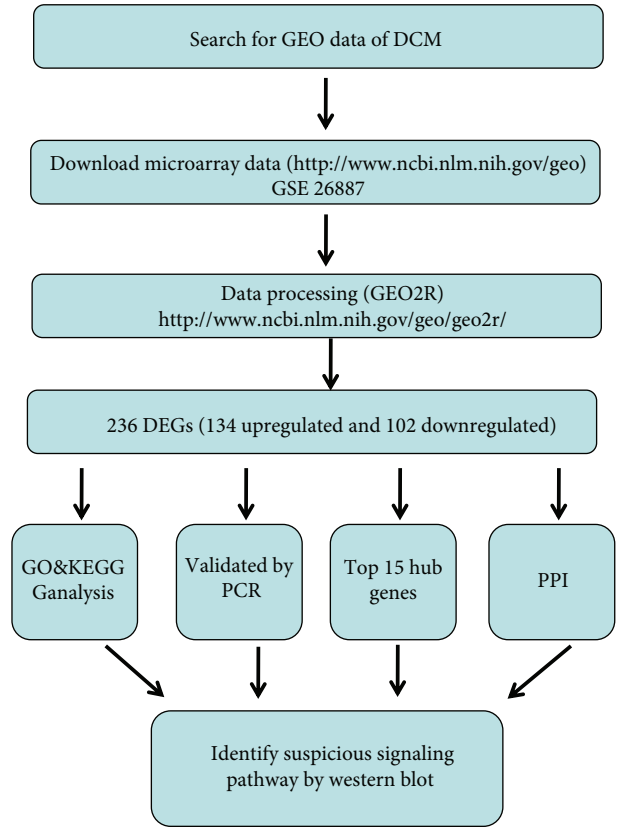


Figure 1: Flow diagram of the analysis procedure: data collection, preprocessing, analysis, and validation.

indicated that the DCM model of db/db mice was constructed successfully (Figure S1A–B). The results from PCR demonstrated that the mRNA expression levels of NPPA, SFRP4, DSC1, NEB, and FRZB were significantly higher in the DCM group compared to the healthy group while the mRNA expression levels of SERPINE1, SERPINA3, ANKRD2, XRCC4, and S100A8 in the DCM group were statistically lower than those in the healthy group (Figures 3(a)–3(j)). Also, we detected the expression levels of these genes in cardiomyocytes and cardiac fibroblasts, respectively. In cardiac fibroblasts, the alterations of the ten genes were consistent with the mouse model (Figure S2A–B). Intriguingly, the expression level of ANKRD2 in cardiomyocytes displayed no significant difference between the normal group and the HG group. In spite of this, other nine genes in cardiomyocytes had a similar variation trend with the mouse model and cardiac fibroblasts (Figure S2C–D). One the one hand, these results increased the credibility of this microarray. On the other hand, these DEGs with the most significant difference may be the promising candidates in clinics to diagnose DCM.

3.2. GO Enrichment Analysis. The results from GO term enrichment analysis varied from expression alterations and GO classification of these DEGs. By analyzing GO enrichment of these upregulated DEGs and downregulated DEGs via DAVID, we discovered that the upregulated DEGs in BP were mainly enriched in the G-protein-coupled purinergic

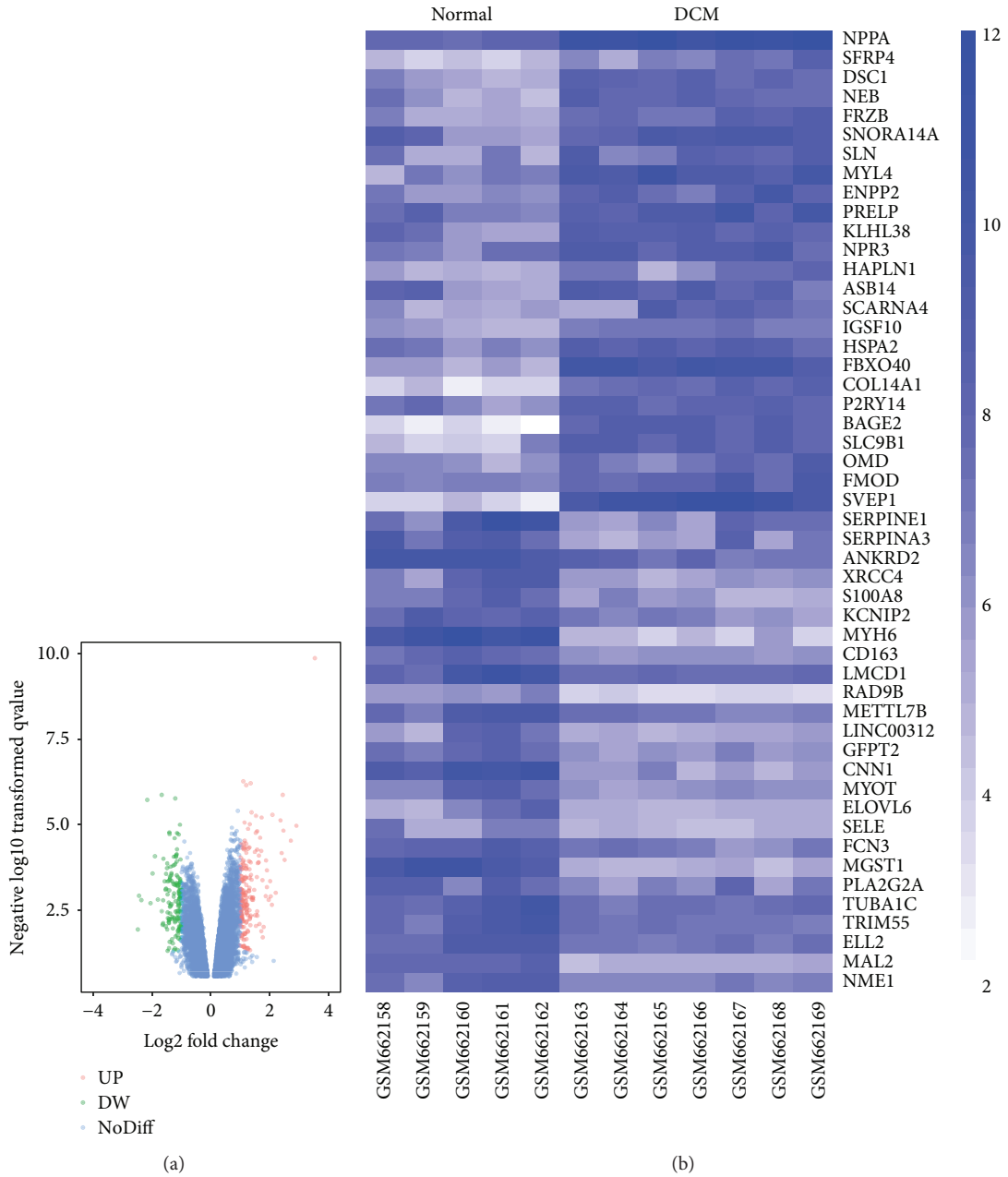


Figure 2: Volcano plot and heat map of the differentially expressed genes (DEGs) between normal samples and patients with diabetic cardiomyopathy (DCM). (a) Volcano plot of genes detected in DCM. Green means downregulated DEGs; red means upregulated DEGs; blue means no difference. (b) Heat map of top 25 upregulated DEGs and top 25 downregulated DEGs.

nucleotide receptor signaling pathway, fatty acid metabolism, mitochondrial membrane potential, extracellular matrix organization, and mitochondrial permeability transition while the downregulated DEGs in BP were enriched in inflammatory response, lipid intake, response to drug, immune response, and platelet degranulation. As for CC, the upregulated DEGs were principally enriched in the integral component of the membrane, plasma membrane, extracellular exosome, extracellular space, and extracellular region while the downregulated DEGs were enriched in the plasma membrane, extracellular space, extracellular region, extracellular exosome, and endoplasmic reticulum membrane. Additionally, MF analysis uncovered that the upregulated DEGs were principally enriched in zinc ion binding, calcium ion binding, heparin binding, collagen binding, and NADP binding. The downregulated genes were responsible for protein binding, mitochondrial uncoupling, cytokine activity, actin binding, and phosphatase activity (Table 2 and Figures 4(a)–4(c)).

3.3. KEGG Pathway Analysis. To acquire more comprehensive information regarding the vital pathways of those selected DEGs, KEGG pathways were also analyzed via DAVID. The results in Table 3 and Figure 4(d) unveiled

Table 1: The top 5 upregulated and downregulated differentially expressed genes in patients with diabetic cardiomyopathy.

DEGs	Gene title	Gene symbol	LogFC	Biological function
Upregulated	Natriuretic peptide A	NPPA	3.53	Extracellular fluid volume and electrolyte homeostasis
	Secreted frizzled related protein 4	SFRP4	2.71	Soluble modulators of Wnt signaling
	Desmocollin 1	DSC1	2.44	Calcium-dependent glycoprotein
	Nebulin	NEB	2.4	Cytoskeleton
	Frizzled-related protein	FRZB	2.36	Soluble modulators of Wnt signaling
Downregulated	Serpin family E member 1	SERPINE1	−2.48	Inhibitor of fibrinolysis
	Serpin family A member 3	SERPINA3	−2.44	Anti-inflammatory and antioxidant effects
	Ankyrin repeat domain 2	ANKRD2	−2.16	Modulator of NF-κB-mediated inflammatory
	X-ray repair cross-complementing 4	XRCC4	−2.05	DNA repair
	S100 calcium-binding protein A8	S100A8	−1.98	Regulating inflammation and oxidative stress, activatingTLR4 signaling

the most important KEGG pathway of the downregulated and upregulated DEGs. The downregulated DEGs were mainly enriched in the PI3K-Akt signaling pathway, MAPK signaling pathway, HIF-1 signaling pathway, TNF signaling pathway, and Toll-like receptor signaling pathway. By contrast, the upregulated DEGs, namely, FMO4, FMO2, FMO3, ADH1B, and UGT2B4, had a strong correlation with drug metabolism-cytochrome P450.

3.4. PPI Analysis. Applying the STRING online tool, 120 nodes with 162 PPI relationships were identified, which accounted for approximately 90.3% of these selected DEGs (Figure 5(a)). Based on the degree of connectivity, we constructed the PPI network and selected the top 15 hub genes (Table 4). The top 15 hub genes, possessing high degree of connectivity in DCM, are as follows: IL6, MYC, ACTA2, SERPINE1, ASPN, SPP1, KIT, TFRC, FMOD, PDE5A, MYH6, FPR1, C3, CDKN1A, and SOCS3. Among these 15 hub genes, IL6, MYC, SERPINE1, SPP1, TFRC, MYH6, FPR1, C3, CDKN1A, and SOCS3 were significantly downregulated while ACTA2, ASPN, KIT, FMOD, and PDE5A were upregulated. The 15 hub genes could interact with 189 genes directly, and IL6 acted as the most intensive gene which could interact with 32 downregulated genes and 15 upregulated genes. Intriguingly, among these, hub genes also displayed strong interactions (Figure 5(b)). For instance, ACTA2 could directly interact with multiple genes (FMOD, IL6, MYH6, MYC, and ASPN), and SPP1 interacted with 4 hub genes (KIT, IL6, MYC, and SERPINE1). The details of the interactions among these 15 hub genes are shown in Table 5. Taken together, these results suggested that these hub genes, especially IL6, ACTA2 as well as SPP1 may exert critical effects in DCM.

3.5. Functional Analysis. To figure out the role of the IL-6/STAT3/SOCS3 signaling pathway in the development of DCM, we detected the protein expression levels of SCOS3, phosphorylated-STAT3 (P-STAT3), and total STAT3 between the normal group and the db/db group. The results showed that P-STAT3 had a significantly higher expression level in the DCM group compared to the normal group. Meanwhile, the level of SCOS3 was significantly downregulated in the DCM group (Figure 6). To further explore the role of the IL-6/STAT3/SOCS3 signaling pathway in cardiomyocytes and cardiac fibroblasts stimulated by HG, we firstly detected the mRNA expression of SOCS3 in cardiomyocytes and cardiac fibroblasts. As expected, the levels of SOCS3 in both cardiomyocytes and cardiac fibroblasts were significantly lower in the HG group compared with the normal group (Figures 7(a) and 7(b)). Additionally, immunofluorescent staining showed that the hypertrophic reactions of cardiomyocytes and phenotypic switching of cardiac fibroblasts were significantly abolished after SOCS3 was upregulated (Figures 7(c) and 7(d)). Meantime, hypertrophic markers and fibrotic markers were also decreased by the overexpression of SOCS3, evidenced by the lower levels of ANP, BNP, collagen I, and collagen III (Figures 7(e) and 7(f)). Western blot showed that the overexpression of SOCS3 could inhibit the phosphorylation of STAT3 (Figures 7(g) and 7(h)).

4. Discussion

Diabetes mellitus has been broadly regarded as one of the leading causes of morbidity and mortality for several decades worldwide. According to estimates, by 2030, there will be approximately 450 million persons with diabetes. DCM serves as the major etiological factor and death cause of patients with diabetes, the incidence of which has increased over recent years [21]. However, currently, there is no specific and efficient diagnostic methodology and treatment strategy for DCM, which is partially because of the complicated molecular mechanisms, as well as its being asymptomatic for the first several years [2]. Hence, some key diagnostic biomarkers and therapeutic targets in plasma and myocardial biopsy should be verified as early as possible. Although myocardial biopsy is not as routine as that in tumors, it does not mean that it makes no sense to perform myocardial biopsy. Takeishi and Yoshihisa retrospectively analyzed 378 patients with suspected cardiomyopathy who underwent myocardial biopsy and found that the diagnostic impact of myocardial biopsy may be relatively high in patients with suspected hypertrophic cardiomyopathy compared to those with suspected dilated cardiomyopathy [22]. Additionally, in patients with

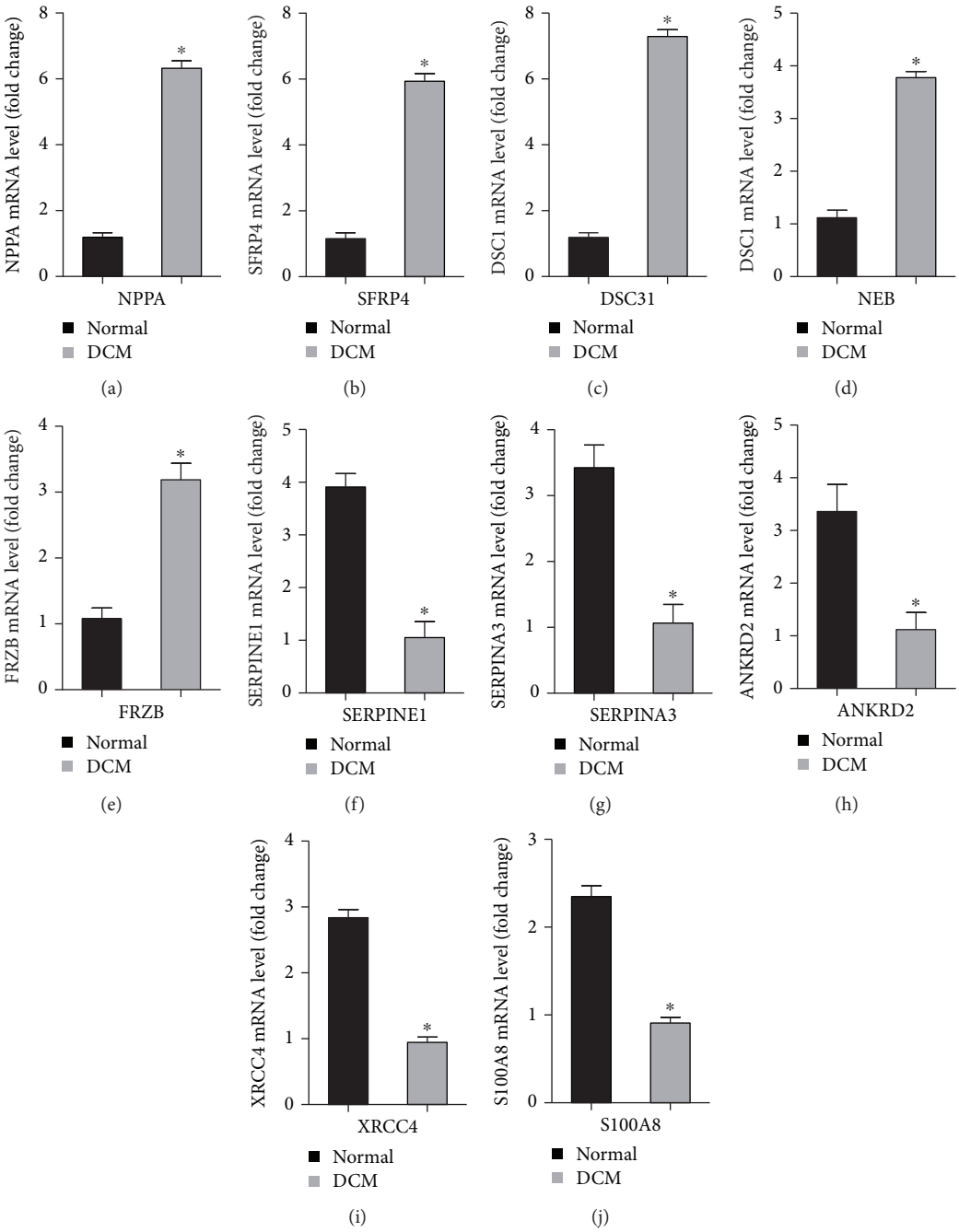


Figure 3: Validation of top 5 upregulated and top 5 downregulated DEGs in the mouse model of DCM. (a–e) NPPA, SFRP4, DSC31, NEB, and FRZB were significantly upregulated in the DCM group. (f–j) SERPINE1, SERPINA3, ANKRD2, ANKRD2, and S100A8 were significantly downregulated in the DCM group. $^*P < 0.05$ versus normal group.

arrhythmogenic right ventricular cardiomyopathy (ARVC), Yoshida et al. detected the expression of plakoglobin and connexin 43 in myocardial biopsy specimens and confirmed the correlations between the levels of these 2 proteins and the development of ARVC, indicating that plakoglobin and connexin 43 are two specific biomarkers of arrhythmic events in ARVC [23]. Furthermore, the combination of cardiac magnetic resonance (CMR) imaging and myocardial biopsy may also improve the diagnostic value in the evaluation of cardiomyopathic conditions [24]. The above studies further supported the potential of myocardial biopsy in diagnosis of DCM. In this study, we firstly performed a comprehensive investigation on expression profiling of myocardial tissue obtained from patients with DCM. Our study included 5 normal individuals and 7 patients with DCM from the GEO database of GSE26887. In our analysis, a total of 236 DEGs (accounting for 2.6% of all genes) were found, involving 134 upregulated genes and 102 downregulated genes. By

Table 2: Gene ontology analysis of differentially expressed genes in patients with diabetic cardiomyopathy.

Expression	Category	Term	Count	%
Upregulated	GOTERM_BP_DIRECT	GO:0035589~G-protein-coupled purinergic nucleotide receptor signaling pathway	9	0.05
	GOTERM_BP_DIRECT	GO:0007155~fatty acid metabolism	8	0.05
	GOTERM_BP_DIRECT	GO:0001501~mitochondrial membrane potential	6	0.04
	GOTERM_BP_DIRECT	GO:0030198~extracellular matrix organization	6	0.04
	GOTERM_BP_DIRECT	GO:0007409~mitochondrial permeability transition	5	0.03
	GOTERM_CC_DIRECT	GO:0016021~integral component of membrane	42	0.25
	GOTERM_CC_DIRECT	GO:0005886~plasma membrane	37	0.22
	GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	29	0.17
	GOTERM_CC_DIRECT	GO:0005615~extracellular space	22	0.13
	GOTERM_CC_DIRECT	GO:0005576~extracellular region	20	0.11
	GOTERM_MF_DIRECT	GO:0008270~glucose transporter	13	0.08
	GOTERM_MF_DIRECT	GO:0005509~calcium ion binding	11	0.07
	GOTERM_MF_DIRECT	GO:0008201~heparin binding	7	0.05
	GOTERM_MF_DIRECT	GO:0005518~collagen binding	5	0.03
	GOTERM_MF_DIRECT	GO:0050661~NADP binding	4	0.03
Downregulated	GOTERM_BP_DIRECT	GO:0006954~inflammatory response	11	0.07
	GOTERM_BP_DIRECT	GO:0008284~lipid intake	9	0.06
	GOTERM_BP_DIRECT	GO:0042493~response to drug	6	0.04
	GOTERM_BP_DIRECT	GO:0006955~immune response	6	0.04
	GOTERM_BP_DIRECT	GO:0002576~platelet degranulation	5	0.04
	GOTERM_CC_DIRECT	GO:0005886~plasma membrane	31	0.21
	GOTERM_CC_DIRECT	GO:0005615~extracellular space	23	0.15
	GOTERM_CC_DIRECT	GO:0005576~extracellular region	21	0.14
	GOTERM_CC_DIRECT	GO:0070062~extracellular exosome	21	0.14
	GOTERM_CC_DIRECT	GO:0005789~endoplasmic reticulum membrane	13	0.09
	GOTERM_MF_DIRECT	GO:0005515~protein binding	58	0.39
	GOTERM_MF_DIRECT	GO:0008083~mitochondrial uncoupling	5	0.03
	GOTERM_MF_DIRECT	GO:0005125~cytokine activity	5	0.03
	GOTERM_MF_DIRECT	GO:0003779~actin binding	5	0.03
	GOTERM_MF_DIRECT	GO:0016791~phosphatase activity	3	0.03

GO: gene ontology.

further annotating and analyzing these DEGs, we identified 10 sensitive biomarkers and top 15 hub genes among these DEGs. Additionally, we also speculated the putative mechanisms of SOCS3 contributing to DCM by Western blot.

4.1. The Production of IL6 Is Essential for the Development of DCM. IL6 is a critical cytokine exerting multiple physiological effects in inflammation and immune regulation, which could be secreted by a range of cell types including monocytes, mast cells, lymphocytes, macrophages, endothelial cells, keratinocytes, tumor cell lines, and fibroblasts [25]. In the innate immune system and adaptive immunity, IL6 stimulation could trigger different biological activities [26]. For example, in innate immunity, IL6 could accelerate the production of neutrophils as well as the synthesis of acute-phase proteins, thus giving rise to acute-phase response while in adaptive immunity, IL6 stimulation could increase the proliferation of B cells [27]. Notably, IL-6 pretreatment increased collagen production in cultured cardiac fibroblasts and promote interstitial fibrosis in Ang II-induced rat heart [28, 29]. Zhang et al. demonstrated that deletion of IL-6 preserved cardiac function and mitigated the interstitial fibrosis in streptozotocin-induced diabetic mice, the mechanism of which may involve the inhibitory effects of IL-6 on TGFβ1 and miR-29 pathway [30]. Clinical trials also disclosed a strong correlation between elevated levels of circulating IL6 and heart failure severity and mortality in patients [31]. In our study, IL6 was an upregulated hub gene with the highest connectivity degree, indicating that IL6 may exert a core and predominant role in the development of DCM. Additionally, according to KEGG analysis, IL6 was significantly enriched in the PI3K/Akt signaling pathway, hypoxia-inducible factor-1 (HIF-1) signaling pathway, TNF signaling pathway, and Toll-like receptor signaling pathway. The previous study has demonstrated that HIF-1 deregulation during the early stage of diabetes gave rise to the development of DCM

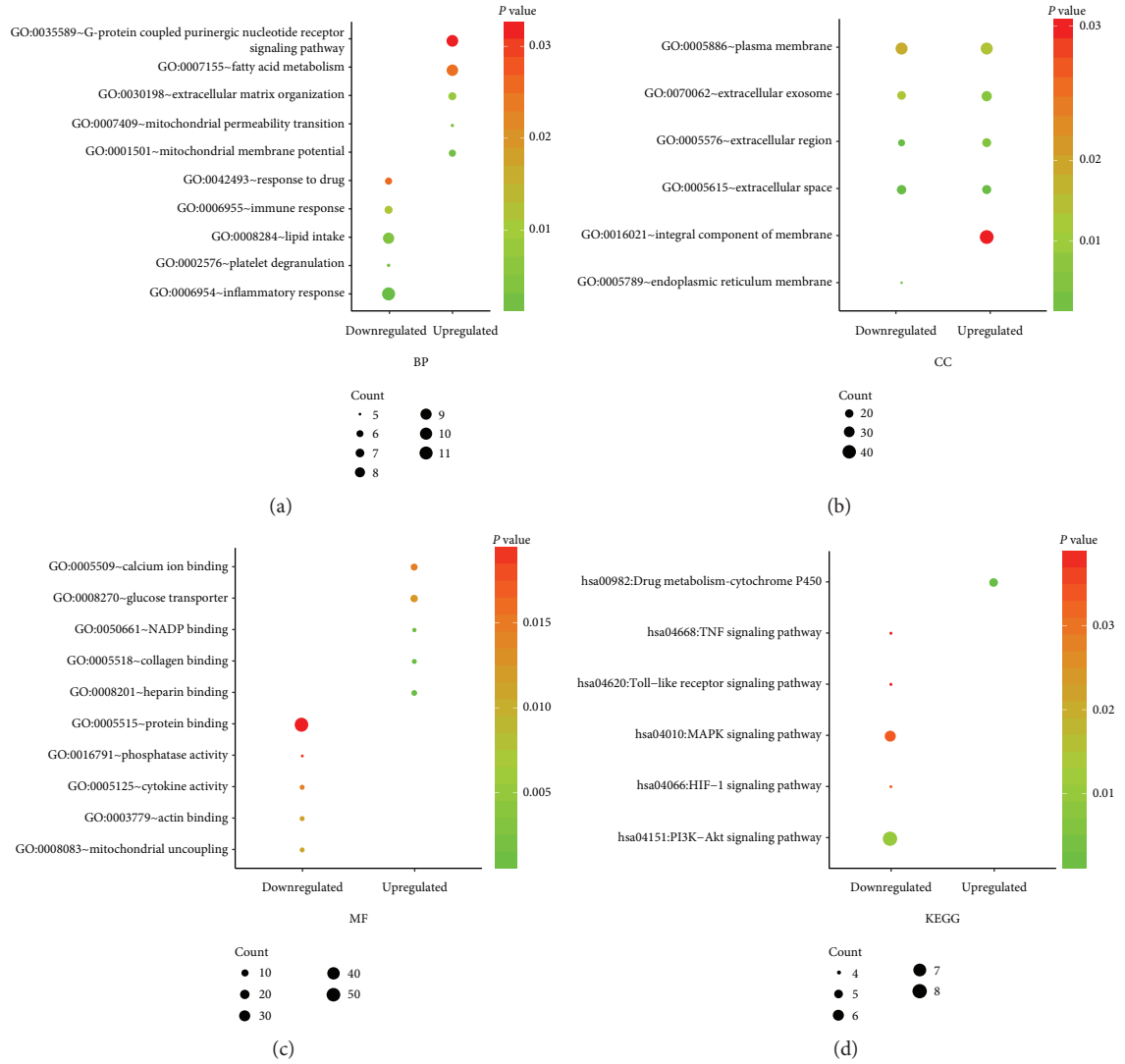


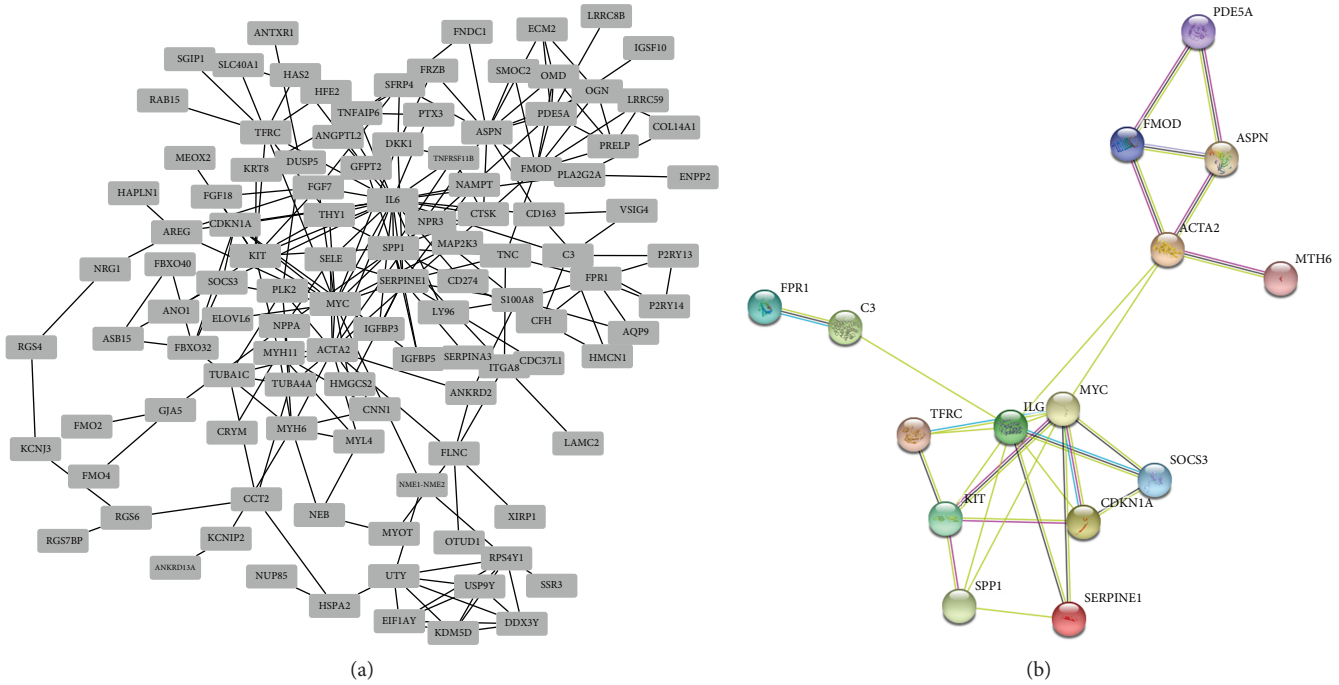
Figure 4: Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DCM. (a) The enriched GO terms in the biological process (BP); (b) the enriched GO terms in the cellular component (CC); (c) the enriched GO terms in the molecular function (MF); (d) the enriched KEGG pathway in DCM.

Table 3: KEGG pathway analysis of differentially expressed genes in patients with diabetic cardiomyopathy.

Category	Term	Count	%	*P* value	Genes
Downregulated DEGs	hsa04151:PI3K-Akt signaling pathway	8	0.05	0.01	FGF18, CDKN1A, IL6, FGF7, TNC, LAMC2, MYC, and SPP1
	hsa04010:MAPK signaling pathway	6	0.04	0.03	DUSP5, FGF18, FGF7, MAP2K3, FLNC, and MYC
	hsa04066:HIF-1 signaling pathway	4	0.02	0.03	CDKN1A, IL6, and TFRC
	hsa04668:TNF signaling pathway	4	0.02	0.04	IL6, SOCS3, MAP2K3, and SELE
	hsa04620:Toll-like receptor signaling pathway	4	0.02	0.04	IL6, LY96, MAP2K3, and SPP1
Upregulated DEGs	hsa00982:Drug metabolism-cytochrome P450	5	0.03	0.001	FMO4, FMO2, FMO3, ADH1B, and UGT2B4

KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: false discovery rate.

[32]. In diabetic retinopathy, the expression of proinflammatory IL6 and TNF-α were significantly inhibited after decreasing the expression of HIF-1 [33]. Hence, whether IL6 could be effectively suppressed via blocking HIF-1 in DCM, eventually alleviating inflammation in myocardium, needs to be further explored.



(a) (b)

Figure 5: Protein-protein interaction (PPI) network. (a) The PPI network of overall DEGs and (b) the PPI network of top 15 hub genes with high connectivity degree.

Table 4: Top 15 hub genes with higher degree of connectivity.

Gene	Degree of connectivity	*P* value
IL6	29	$5.48E-04$
MYC	17	$3.99E-03$
ACTA2	14	$9.78E-06$
SERPINE1	14	$1.16E-02$
ASPN	12	$9.15E-03$
SPP1	11	$4.04E-02$
KIT	11	$4.54E-03$
TFRC	9	$3.34E-04$
FMOD	9	$3.42E-04$
PDE5A	9	$2.96E-04$
MYH6	8	$1.55E-03$
FPR1	8	$1.71E-06$
C3	7	$1.18E-02$
CDKN1A	7	$3.94E-04$
SOCS3	7	$1.02E-03$

4.2. Inflammation and Immune Disorders Are Vital Pathophysiological Alterations of DCM. Another major finding of our study is that the inflammation and immune disorders mediated by cytokines may exert an important role in DCM [34]. Cardiac inflammation is one of the important features in heart failure. Enhanced proinflammatory cytokine expression levels and intensified immune cell infiltration, including macrophages and cytotoxic T lymphocytes, have been previously observed in the inflamed heart in DCM [35]. The activation of certain molecular pathways including c-Jun NH2-terminal kinase, NF-κB, and p38-MAPK could aggravate the development of inflammation which displayed a strong relationship with insulin resistance, thus inducing DCM [36–38]. In the diabetic hearts of type 2 diabetes patients, elevated inflammatory cytokines, such as IL6, TNF-α, cell adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), and acute phase reactants, such as C-reactive protein and other inflammatory markers, have been verified [39, 40]. On the other hand, immune disorder in cardiovascular diseases has been also studied for decades. Particularly, activation of the immune system is not independent of inflammation in the progression of heart failure. In chronic heart failure, activating the immune system can usually contribute to the activation of the complement system, secretion of proinflammatory cytokines, and production and release of autoantibodies [41]. As for DCM, impaired systolic and diastolic LV function in the streptozotocin-induced diabetes, to a great extent, was correlated with increased immune cell invasion and adverse cardiac remodeling [42]. Toll-like receptors (TLRs) are a type of membrane-anchored proteins existing in various cell types involving immune cells (macrophages and lymphocytes) and nonimmune cells (cardiomyocytes) [43, 44]. Cardiac TLRs and inflammasome could interact with each other, inducing inflammation through reactive oxygen species overproduction and NF-κB activation [45]. In our study, the enriched Toll-like receptor signaling pathway was observed from KEGG analysis, indicating the vital role of immunity in DCM. Meanwhile, GO analysis unveiled that the downregulated DEGs were mainly enriched

Table 5: Gene-specific primers used in quantitative real-time PCR.

Species	Genes		Sequences
Mouse	GAPDH	Forward	5′-ACTCCACTCACGGCAAATTC-3′
		Reverse	5′-TCTCCATGGTGGTGAAGACA-3′
Mouse	NPPA	Forward	5′-CCCTCCGATAGATCTGCCCT-3′
		Reverse	5′-GTCAATCCTACCCCCGAAGC-3′
Mouse	SFRP4	Forward	5′-AAAAGCCGTCCAGAGGAGTG-3′
		Reverse	5′-GAGGGACTTGTGTTCGAGGG-3′
Mouse	DSC31	Forward	5′-GATCAGGCCAGTGGAAATGT-3′
		Reverse	5′-GTGTGTTTCGTGCAACCATC-3′
Mouse	NEB	Forward	5′-ATCCTGTCCAAACTAAGGCTCG-3′
		Reverse	5′-ACCTCTTTAGCATAGTAGTCCGC-3′
Mouse	SERPINE1	Forward	5′-GGGTTCACTTTACCCCTCCG-3′
		Reverse	5′-TAGGGCAGTTCCACAACGTC-3′
Mouse	SERPINA3	Forward	5′-TGACCTTTCTCAGCACGACC-3′
		Reverse	5′-AATAGGGGAGGATGGGAGCA-3′
Mouse	ANKRD2	Forward	5′-TTGCCCAGGAGGAAGAGACT-3′
		Reverse	5′-TGTCTCTCACGTTGGTGTCG-3′
Mouse	XRCC4	Forward	5′-TTGGGCGCATCGGTTTATCT-3′
		Reverse	5′-ACCAGTGCCTTTCTCAGCTC-3′
Mouse	S100A8	Forward	5′-TTCGTGACAATGCCGTCTGA-3′
		Reverse	5′-GGCCAGAAGCTCTGCTACTC-3′

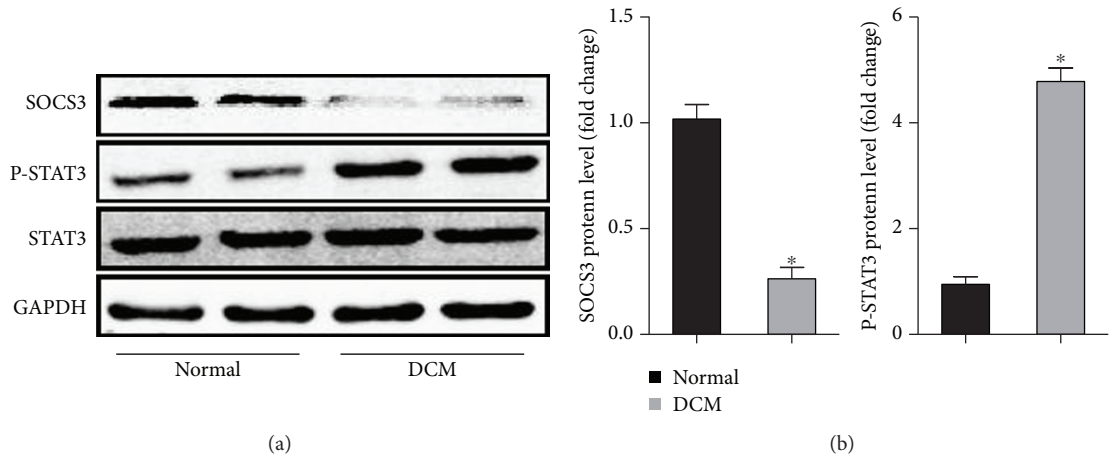


Figure 6: Identification of the STAT3/SOCS3 pathway in an in vivo model of DCM. SOCS3: phosphorylated-STAT3 (P-STAT3) and total STAT3 protein levels as shown by Western blot analysis.

in inflammatory response and immune response. Additionally, we found that the upregulated DEGs were significantly enriched in the G-protein-coupled purinergic nucleotide receptor signaling pathway in BP. To our knowledge, the nucleotides, the fundamental subunits of nucleic acids, are released by mast cells, macrophages, T cells, endothelial cells, and platelets in response to physiological activation. The purinergic nucleotide receptor could inhibit the activation of effector T cells in many allergic diseases [46]. Thus, we hypothesize that balancing the immune homeostasis by regulating the nucleotide receptor signaling pathway may be promised to be a novel strategy to treat DCM. Last but not

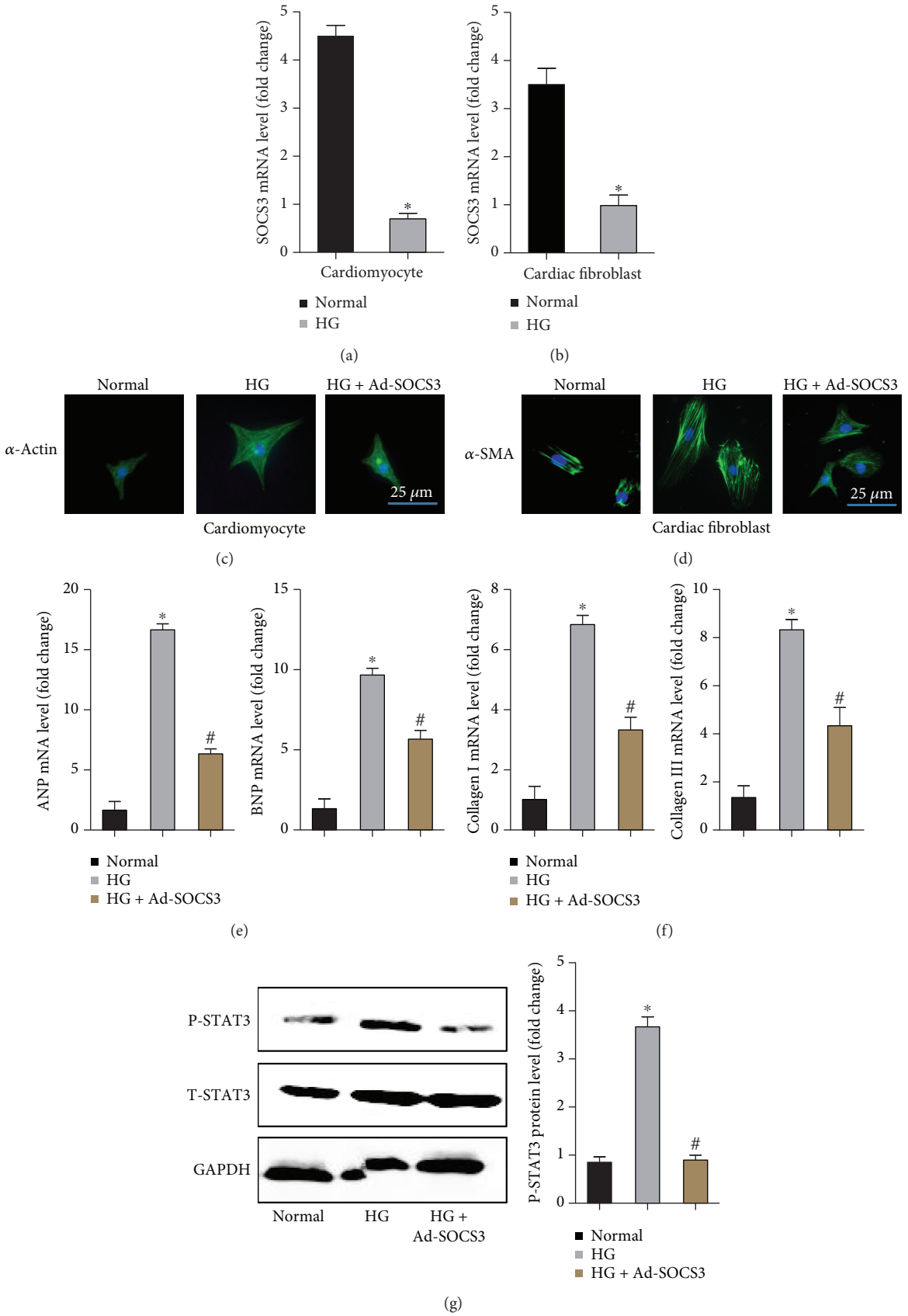


Figure 7: Continued.

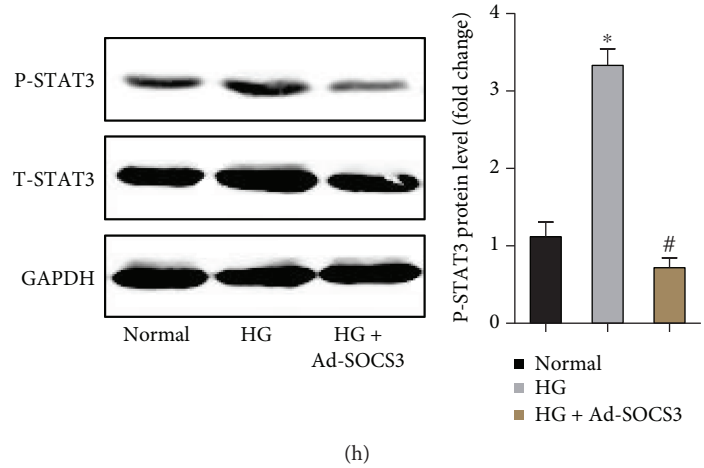


Figure 7: The expression and effects of SOCS3 in cardiomyocytes and cardiac fibroblasts. (a) The mRNA level of SOCS3 in cardiomyocytes induced by HG. (b) The mRNA level of SOCS3 in cardiac fibroblasts induced by HG. (c) The immunofluorescence staining of α-actin in cardiomyocytes induced by HG with or without SOCS3 overexpression. (d) The immunofluorescence staining of α-SMA in cardiac fibroblasts induced by HG with or without SOCS3 overexpression. (e) The mRNA levels of ANP and BNP in cardiomyocytes induced by HG with or without SOCS3 overexpression. (f) The mRNA levels of collagen I and collagen III in cardiac fibroblasts induced by HG with or without SOCS3 overexpression. (g, h) The protein expression of P-STAT3 and T-STAT3 in the indicated groups. $^{*}P < 0.05$ versus normal group and $^{\#}P < 0.05$ versus HG group.

least, many of the hub genes and top 5 downregulated or upregulated genes were also implicated with inflammation and immune response: SERPINA3 (immune response to elevated platelet cytosolic Ca^{2+}), S100A8 (regulating inflammation and oxidative stress, activatingTLR4 signaling), ANKRD2 (a modulator of NF-κB-mediated inflammatory), FPR1 (G protein-coupled receptor, inflammation), C3 (immune response), CDKN1A (inflammatory response gene), SOCS3 (regulating interleukin), and IL6 (proinflammatory cytokine).

Taken together, our results, in the perspective of bioinformatics, provide compelling evidence for the fact that inflammation and immune effects play critical roles in the development in DCM. Sincerely, we hope that these findings could provide new strategies and insights to identify the pivotal targets and pathways with regards to the immunologic mechanisms of DCM in future research.

4.3. The Improvement of Cardiac Metabolism and Calcium Homeostasis May Benefit DCM. Cardiac metabolic abnormalities are broadly recognized to increase various death risks. In diabetic heart, glucose oxidation was significantly decreased while the intake of fatty acids and its oxidation rate were further elevated. Increased dependence on fatty acids to generate energy may predispose the diabetic heart to the endoplasmic reticulum, oxidative stress, stress, and ischemic damage. Accumulation of intracellular toxic lipid metabolites gives rise to a great many cellular abnormalities resulting in cardiac dysfunction and cardiac remodeling [47]. Meanwhile, the abnormal mitochondrial membrane potential and permeability not only increased the production of reactive oxygen species but also impaired its elimination, causing accumulation of reactive oxygen species in diabetic heart. Excessive production of reactive oxygen species and loss of endothelial antioxidant barrier scales could lead to the production of oxidative stress. Additionally, calcium ion could enter the mitochondrial matrix mediated by a calcium uniporter complex in a sodium calcium exchanger manner, which helps to alleviate calcium overload. In our study, the GO analysis demonstrated that the DEGs were primarily enriched in fatty acid metabolism, mitochondrial membrane potential, mitochondrial uncoupling, glucose transporter, calcium ion binding, and lipid intake.

4.4. SOCS3 May Act as a Novel Therapeutic Target in DCM. The role of the IL-6/STAT3/SOCS3 signaling pathway in tumors has been well evaluated. IL6 stimulates survival, proliferation, and progression to cancer of intestinal epithelial cells via activation of signal transducers and activators of transcription 3 (STAT3), eventually inducing the expression of SCOS3 [47]. Meanwhile, SOCS3 is an important inhibitory factor of STAT3, which could block the phosphorylation of STAT3 and negatively regulate IL-6/STAT3 signaling [25]. Under normal circumstances, the activation of STAT3 is transitory and speedy, while STAT3 could be constitutively activated under pathologic status, which is attributed to the absence or downregulation of SOCS3 [48]. Currently, the role of SCOS3 in DCM remains unclear. We found that both IL6 and SOCS3 acted as hub genes in our study. Therefore, we put forward a hypothesis that IL6 trans-signaling may also activate STAT3/SOCS3, thus promoting the development of DCM. As expected, Western blotting showed that the protein expression of SOCS3 was significantly lower in the DCM group than that in the control group while the phosphorylation of STAT3 was significantly higher in the DCM group, indicating the inhibitory effects of SOCS3 on the phosphorylation of STAT3. To obtain more accurate results, further studies should be performed to explore the

accurate mechanisms of the IL-6/STAT3/SOCS3 signaling pathway in DCM.

5. Conclusion

Conclusively, using a series of bioinformatics analysis, we give a comprehensive and novel illustration of gene expression profiles to identify DEGs expressing in myocardial tissue, which may play critical roles in the occurrence and development in patients with DCM. Genes and pathways implicated with inflammation, immune, and metabolism were significantly altered in DCM. Notably, IL6 may act as a much more important role in the development of DCM beyond our expectation. Additionally, targeting the IL-6/STAT3/SOCS3 signaling pathway is a promising strategy to treat DCM. These findings will greatly contribute to unveiling the molecule mechanisms of DCM. To allow these biomakers and targets to be used more routinely in clinic, further investigations into the correlation of plasma proteins as well as metabolites and these dysregulated genes should be performed.

Data Availability

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

Conflicts of Interest

No conflict of interest exists.

Authors' Contributions

Ning Li and Haiming Wu, and Rongxin Geng contributed equally to this work.

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Supplementary Materials

Supplementary 1. Supplemental Table S1: gene-specific primers used in quantitative real-time PCR.

Supplementary 2. Figure S1: the construction of DCM in db/db mice. (A) Representative echocardiographic images in the control group and DCM group. (B) Representative images of the H&E staining and PSR staining in the indicated group.

Supplementary 3. Figure S2: the validation of top 5 upregulated and top 5 downregulated DEGs in vitro. (A) The mRNA expression of NPPA, SFRP4, DSC31, NEB, and FRZB in cardiomyocytes. (B) The mRNA expression of SERPINE1, SERPINA3, ANKRD2, ANKRD2, and S100A8 in cardiomyocytes. (C) The mRNA expression of NPPA, SFRP4, DSC31, NEB, and FRZB in cardiac fibroblasts. (D) The mRNA expression of SERPINE1, SERPINA3, ANKRD2, ANKRD2, and S100A8 in cardiac fibroblasts. $^{*}P < 0.05$ versus normal group.

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Review Article

Usefulness of the Adipokines as Biomarkers of Ischemic Cardiac Dysfunction

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Cardiovascular disease is the leading cause of death among both women and men, but there is still a great percentage of misdiagnosis and lack of clearly defined criteria. Advances in biomolecular science have proven the crucial role of inflammation and, more importantly, the role of adipokines in mediating all stages of coronary artery disease. It has also been suggested that regional fat deposits, more precisely from thoracic region, have a major influence on the development of coronary artery disease by creating a local proatherogenic environment. The immune system closely interacts with metabolic risk factors to initiate, promote, and further aggravate the atherosclerotic lesions on the arterial wall all with the "help" of adipokines. So nowadays, research extensively focuses on uncovering biomarkers that would provide an increased chance of detecting subclinical cardiac distress and also add a consistent value to current guideline-imposed risk criteria.

1. Introduction

The adipose tissue secretes several hormone-like molecules called adipokines, which participate in body physiology regulation. Adipokines, pleiotropic molecules, also play a role in diseases such as diabetes, atherosclerosis, and autoimmune diseases, among others [1]. The adipokine group includes classical cytokines (e.g., tumor necrosis factor-α (TNFα), interleukin-6 (IL-6)), specific chemokines (interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-2α (MIP-2α), stromal cell-derived factor-1 (SDF-1)), growth factors (e.g., transforming growth factor-β (TGF-β)), and proteins of the alternative complement system (e.g., adipsin, acylation-stimulating protein). The group also includes proteins involved in vascular hemostasis (e.g., plasminogen inhibitor activator-1 (PAI-1), tissue factor), lipid metabolism (leptin, retinol-binding protein, and cholesteryl ester transfer protein), glucose homeostasis (e.g., adiponectin, resistin), and angiogenesis (e.g., vascular endothelial growth factor (VEGF)), as well as acute phase and stress responses (e.g., haptoglobin, metallothionein) [2, 3]. Production of these proteins by adipose tissue is increased in obesity and has led to the idea that obese patients are characterised by a state of chronic low-grade inflammation, that links causally to insulin resistance and the metabolic syndrome. Figure 1 describes the role of obese adipocytes in the inflammation and pathogenesis of atherosclerosis [4].

There is no doubt that inflammation is viewed as an important pathophysiological step in the development of atherosclerosis. There are a multitude of studies that address this issue and also the issue of the interaction between the immune mechanisms and metabolic risk factors. This interaction initiates, promotes, and activates the lesions in the coronary arteries, and currently, efforts are made to find the key biomarkers that could unveil the risk of the progression of atherosclerosis in patients with metabolic disorders.

2. Adipokines as Biomarkers in Atherosclerotic Heart Disease

2.1. Biomarker Definition. A biomarker is a "characteristic that is objectively measured and evaluated as an indicator

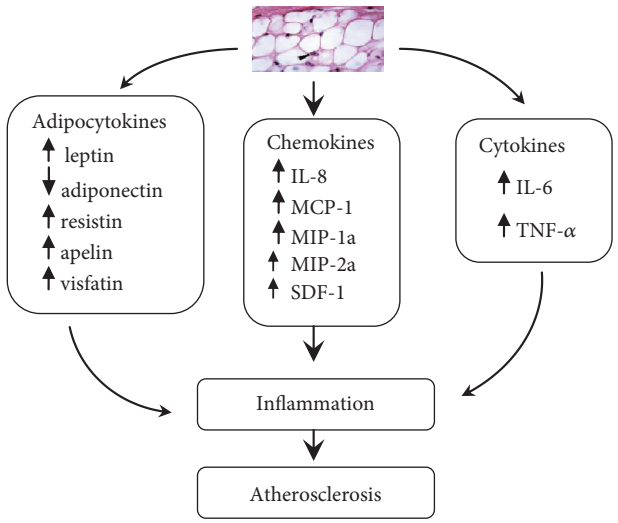

Figure 1: The role of obese adipocytes in the inflammation and pathogenesis of atherosclerosis (adapted from Opatrilova et al. [4]).

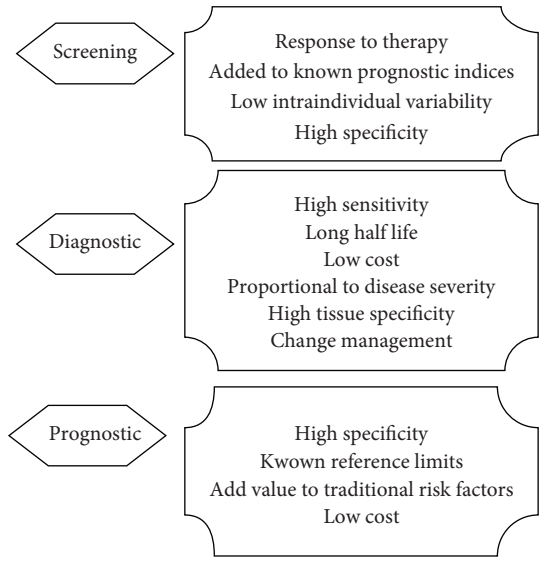

Figure 2: Ideal biomarker characteristics (adapted from Zhao et al. [9]).

of the normal biological process, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [5]. An ideal biomarker should be used for screening, prognostic, and diagnostic purposes (Figure 2).

Recent research has shown that medicine needs precision, and it is important to have in mind that people are different with respect to genes, environment, and lifestyle factors and that treatments need to be more effective and targeted. This is one of the reasons that we need prognostic, pharmacodynamic, or predictive biomarkers [6, 7].

When introducing a new biomarker, one has to keep in mind that this novel molecule needs to be as close as possible to an "ideal biomarker" regarding accuracy and standardization of determination, reproducibility, accessibility, high sensibility, and specificity and it should also make an impact in the clinical care of a patient [8].

When interpreting the serum levels of a biomarker, one should refer to the following:

(i) Reference limits: these are cut-off values statistically established taking into consideration disease-free individuals

(ii) Discriminative limits: these are the limits that impose a decision. For example, if we take into account troponin, the 99 percentile is discriminative for acute myocardial infarction so that we can correctly assess patients with acute myocardial infarction apart from healthy individuals

(iii) Risk limit: this is the limit from where we consider a greater risk

At the present time, there are only a few number of established biomarkers (e.g., NT-proBNP, BNP, cTnI, cTnT, CRP, and D-dimers) but important research is being conducted in developing new ones as the diagnostic, prognostic, and therapeutic impact is remarkable.

2.2. Classification of Biomarkers in Cardiovascular Disease. There is a large number of biomarkers evaluated or under evaluation in relation with cardiovascular disease, and detailing all of them exceeds the purpose of this paper. But, if we strictly refer to atherosclerotic disease, they can be grouped as follows [6, 10]:

(i) Biomarkers for acute changes: copeptin, high sensitivity troponin, galectin-3, ST-2, heart fatty acid-binding protein (H-FABP), pregnancy-associated plasma protein-A (PAPP-A), ischemia-modified albumin (IMA)

(ii) Biomarkers for chronic changes: coronary calcium

(iii) Biomarkers of inflammation: C-reactive protein, interleukin-6, fibrinogen, monocyte chemotactic protein-1, TNF-alpha, myeloperoxidase, soluble fragment CD40 ligand (sCD40L), angiotensin II, E-selectin, heat shock proteins, matrix metalloproteinases (MMP), myeloperoxidase (MPO), platelet endothelial cell adhesion molecule-1 (PECAM-1), intracellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1)

(iv) Biomarkers of metabolic disorders: lipoprotein (a), low-density lipoproteins, high-density lipoproteins, apoB100, lipoprotein-associated phospholipase A2, homocysteine, adiponectin, haptoglobin, visfatin, leptin, resistin, etc.

Some of the above listed are already documented as biomarkers, so the present paper refers to those that derive from the adipose tissue and that are still under research but with promising results, which we have also confirmed in our studies.

2.2.1. Visfatin in Atherosclerosis. Visfatin is a new identified adipokine that plays a role as a proinflammatory mediator

in the process of atherosclerosis and also in plaque destabilization, also known as NAMPT (nicotinamide phosphoribosyltransferase) and PBEF (pre-beta cell-enhancing factor). Research has shown that this adipokine has insulin mimetic features and is involved in the process of pancreatic pre-beta cell maturation. It was first described in 2005 by Fukuhara and his collaborators [11]. These authors showed that visfatin correlates with visceral fat. Even though their research paper was partially retracted due to technical issues, mainly, the multiple functions of this cytokine were confirmed in an increasing number of studies. Also, it was demonstrated that bariatric surgery in morbidly obese patients helped lower the plasma level of visfatin 6 months afterwards [12]. Obesity and T2DM are independent risk factors for atherosclerotic disease, and it seems that plasma levels of visfatin are increased in this set of subjects. Kadoglou et al. demonstrated this in 2010, in a study conducted on 120 diabetic patients without clinical vascular complications, compared with age- and sex-matched healthy individuals [13].

Visfatin acts on a large number of sites as follows [14]:

(i) Endothelial wall: angiogenesis, tumor growth, cardiac fibrosis

(ii) Cardiomyocytes

(iii) Vascular tone: in healthy subjects, it promotes vasodilation, but in subjects with T2DM, it promotes vasoconstriction

(iv) Extracellular matrix: it determines augmentation in MMP2/9 release which promotes plaque weakening

As it is well known by now, arterial hypertension, dyslipidemia, smoking, and T2DM determine repetitive lesions on the endothelium of the arterial vessels. This leads to a growing inflammatory response. There are a number of authors that have demonstrated that in the presence of inflammation, visfatin levels are increased. Visfatin may contribute to cardiac fibrosis indirectly via the vascular endothelium and acts at this level (of the endothelium) by superactivating ERK-NT-kB-iNOS axis, thus activating among others, matrix metalloproteinases. Visfatin also enhances the production of myocardial repair tissue (when cardiomyocytes were acutely exposed to it) and remodeling (if cardiomyocytes were chronically exposed to it) via upstreaming matrix metalloproteinase-9 and vascular endothelial growth factor [15]. This is confirmed by demonstrating the superexpression of mRNA. Also, eNAMPT enhances cardiac hypertrophy via calcineurin/NFAT signaling pathway—a genetic mechanism involved in cardiac hypertrophy. Visfatin is also known as NAMPT (nicotinamide phosphoribosyltransferase), and NAMPT has the role as an NAD+-synthesizing enzyme within the cell (iNAMPT). This enzyme protects against cardiac hypertrophy by maintaining optimal high levels of NAD+. But when released into the circulation, eNAMPT acts as a proinflammatory adipokine, cytokine, and growth factor and induces endothelial dysfunction and destabilization of atherosclerotic plaque [16].

Therefore, in concern to the involvement of visfatin in the atherosclerotic plaque, this is due to the fact that it acts like an intracellular regulator of NAD+-dependent reactions in the muscle cell. It also promotes vascular inflammation and proliferation in association with atherosclerotic plaque [17].

Until now, the majority of research showed that visfatin might be in relation with obesity but mostly in the presence of T2DM. Hypertension does not seem to alter visfatin plasma levels. Our study, conducted on two different types of hypertensive patients, also confirms these findings [18].

If we were to analyze visfatin, from a biomarker view, one notices that, at this moment, it seems to meet some of the criteria mentioned before:

(i) it has high specificity: values to be found elevated in T2DM with obesity

(ii) it adds value on traditional risk: patients with obesity and T2DM who also had high visfatin plasma levels seem to have more advanced atherosclerosis

With respect to the response to therapy, it was found that lipid-lowering therapy significantly lowers visfatin plasma levels. Also, there are a number of studies that show that inhibiting visfatin might represent a novel therapeutic approach in cardiovascular or cerebrovascular complications of atherosclerosis [19].

2.2.2. Apelin in Atherosclerosis. Apelin is a relatively newly discovered peptide, with various and significant activities within the human body. Initially, it was considered to be an adipocytokine, but it was concluded that its most important actions are directed to the cardiovascular system particularly in the endothelium and myocardium. Both apelin and its receptor, APJ, are highly expressed in the vessels and heart. It is synthetized as preprohormone and then cleaved in fragments of different lengths [20–23]. The majority of its effects are chain links in a complicated and intricate protection mechanism which is designed to regulate basic functions of the organism, aid the regenerative processes, and slow the diseases. It is subject for research in various fields and pathologies, the researchers' attention being focused on apelin-13, the most important apelin isoform for the cardiovascular system [24]. Its protective activity is mainly accomplished through antagonism of the renin-angiotensin-aldosterone system, but other mechanisms are revealed in the latest research [25–28].

This protective effect of apelin is expressed also on the atherosclerotic process and its development [29, 30]. Apelin-13 inhibits the formation of the macrophage foam cells; the main mechanism for that is the activation of the protein kinase C and initiation of some molecular pathways that in the end will significantly reduce the cholesterol level in the macrophage foam cells and the production of foam cells themselves [31]. In this process, the stimulation of autophagy is the key effect [32]. In favor of this result came the observations of Cui et al. about microRNAs; their increased expression has an interesting effect of reducing the expression of apelin in macrophages concomitant with

increasing the lipid accumulation and lowering the efflux of cholesterol from macrophages. At the same time, the apelin molecules that were resistant to microRNA effects continued to inhibit the accumulation of lipids in the macrophages. According to Cui et al. observations, miRNA-497 is involved in modulating the efflux of oxidized LDL cholesterol from the THP-1 macrophages and apelin is one of the molecule from the downstream molecular chain reactions. Overexpression of miRNA-497 was observed to significantly reduce the expression of apelin in THP-1 macrophages [33]. Enforced expression of miR-497 promoted lipid accumulation and decreased cholesterol efflux in oxLDL-exposed THP-1 macrophages. In contrast, downregulation of miR-497 suppressed oxLDL-induced lipid accumulation in THP-1 macrophages. Overexpression of miR-497 significantly reduced the expression of apelin in THP-1 macrophages. The levels of microRNA studied in this research were increased and negatively correlated with the level of apelin in the atherosclerotic lesions [34].

The experimental studies showed that the administration of apelin-13 results in lower levels of proinflammatory cytokine secretion (interleukin-6, interleukin-1β, and tumor necrosis factor-α) and lower expression of lipoprotein lipase, and so, it favors the reduction of lipid accumulation in the vascular wall [35]. In experimental models of aortic abdominal aneurysm induced by elastase, the exogen apelin administration reduces the content of macrophages within the aortic wall and the chemokine production and in the end, reduces the formation of aortic aneurysm [36].

The initial studies showed that in patients with acute coronary syndromes, the level of apelin lowered as the severity of coronary stenosis increased. Highly unstable atherosclerotic plaques were associated with lower levels of apelin [37, 38]. Furthermore, this peptide appears to be involved in the development of collateral vessels in patients with stable angina; the study of Akboga et al. showed that apelin can be a predictor of collateral vessel development, besides other factors such as the severity of stenosis or the presence of right coronary occlusion or severe multivessel disease. In their research, apelin was an independent predictor of good coronary collateral network.

Although the data is still incomplete, apelin appears to be an atheroprotective factor, and most important, it shows potential for therapeutic manipulation but also for risk stratification. But further studies are needed to clarify both the predictive value of this marker and its therapeutic value.

Atherosclerosis is a major pathology which affects all vital organs and is a cause for severe morbidity and mortality, with increased costs. Any therapeutic modulation of the pathophysiologic process in order to limit the clinical consequences is welcomed, and apelin appears to be an important potential target for treatment. Another potential target could be the adipose tissue macrophage activation as suggested by the paper of Boutens et al. [39].

2.2.3. Leptin in Atherosclerosis. Through the adipocytokines released by the fatty tissue, after a unique metabolic rewiring, the fat tissue influences the inflammation in the whole body, particularly the atherosclerotic lesion development [40]. Leptin is an adipocytokine produced in the adipose tissue with a primary role of regulating the food intake and energetic metabolism, in order to keep the fat tissue at a constant level. It is an anorectic agent through actions on the hypothalamus; because obese patients have a high level of serum leptin, it was thought that the main mechanism in obesity is a resistance to leptin [41].

This adipocytokine has also an important activity on the cardiovascular system. It was shown that it is both atherogenic and antiatherogenic factors, and it appeared to be a predictive marker for cardiovascular events. Its importance as a protective factor is not yet clarified, although it was suggested that hyperleptinemia might not be directly linked to atherogenesis, but it might reflect and be a consequence of a state of leptin resistance. The obesity paradox was described, and it represents only a small part of the complex molecular system that controls and promotes the inflammation and its effect on blood vessels [42–44].

It appears that high levels of leptin promote the production of other inflammatory mediators such as tumor necrosis factor, interleukin-2, and interleukin-6 and increase the production of the reactive oxygen radicals. Leptin stimulates the proliferation and hypertrophy of smooth muscle cells within the vessel wall and the accumulation of cholesterol esters in foam cells, particularly if associated with hyperglycemia [42, 45]. It is well known that all these effects contribute to endothelial dysfunction and promote the development and progression of atherosclerotic lesions, but it is not yet clear if leptin itself can induce the atherosclerotic process or it is only a marker of the biological context in which the vascular lesions are produced.

Leptin was intensely studied in cardiovascular patients, and it was observed that it is predictive for metabolic syndrome, myocardial infarction and coronary events in men and hypertensive women, and ischemic stroke [42, 46]. In combination with adiponectin, another adipocytokine with protective effects for the vessels, leptin is a valuable marker for the prediction of the atherosclerotic process, both their serum values being useful, but more importantly their ratio (leptin/adiponectin) [47].

Leptin, together with adiponectin, is a link in the adipovascular axis, connecting the excessive fat tissue with inflammation and atherosclerosis, and all the pathologies that derive from their imbalance. The leptin/adiponectin ratio is a new and better marker for atherosclerosis (and for monitoring the atherosclerotic index), insulin resistance, and endothelial dysfunction, but gender differences must be kept in mind because of the hormonal influences on the adipose metabolism and adipokine production [47].

2.2.4. Resistin in Atherosclerosis. Human resistin, a 12.5 kDa cysteine-rich polypeptide [48] circulating in different molecular isoforms [49], is expressed at lower levels in adipocytes but at higher levels in circulating monocytes and macrophages [50] and vascular endothelium [51]. It has proinflammatory effects by nuclear factor-kappa B (NF-κB) activation and production of cytokines (IL-6, IL-1, TNF-alpha, and monocyte chemoattractant proteins) [52, 53]. At the same time, it exerts vascular remodeling effects by the stimulation

of angiogenesis [54], proliferation capacity [55], and expression of endothelin-1, VCAM-1, and MCP-1 [56] by vascular smooth muscle cells. Resistin also decreases the production of nitric oxide in coronary endothelial cells and increases the production of reactive oxygen species (ROS) [57].

As a result, resistin stimulates monocytes, endothelial cells, and vascular smooth muscle cells, thereby inducing atherosclerosis in experimental animals [58]. These experimental evidences, together with clinical studies demonstrating the association of resistin plasma levels with obesity [59, 60], metabolic syndrome (MetS) [61], and ischemic heart disease [62, 63], suggest that resistin might play a role in the interaction between insulin resistance, inflammation, and atherosclerosis [59, 60]. Several studies have shown that variants of the resistin gene and their imbalance have an impact on metabolic parameters and cardiovascular risk [64, 65]. Subjects with 420C/G allele showed increases in resistin plasma concentrations [66, 67], elevated glucose levels at birth [68], high HbA1c levels [69], and increased risk of T2DM [65]. At the same time, they also had increased levels of triglycerides and higher prevalence of MetS and obesity [61]. In both females and males, the G allele of the 420C/G of the resistin gene polymorphism appears to be associated [70] with higher risk for cardiovascular events [61], cerebrovascular disease, and stroke [69, 71]: more severe stroke and higher in-hospital mortality in patients with acute ischemic stroke [72]. Similarly, subjects with allele 299A (allele +299 (G>A) alleles) exhibited high glucose levels at birth, and in diabetic patients, the resistin levels correlated with cerebrovascular disease especially in males [72].

Although these characteristics make resistin a good biomarker of atherosclerosis, its relationship with IMT—a marker of initial asymptomatic atherosclerosis—has been reported only in obese children [73] but there is no evidence of this association in obese adults [74]. In contrast, in obese patients, resistin correlated with key components that correlate vascular risk parameters to the development of atherosclerosis: platelet number and volumes (MPV), serum and platelet P-selectin, fibrinogen, PAI-1 ag (plasminogen activator inhibitor-1 antigen), and PMPs (platelet-derived microparticles [16, 46, 74]). At the same time, increased resistin levels found in smokers correlate with insulin resistance, while both in smokers and nonsmokers, they are not related to C-reactive protein, homocysteine, and uric acid levels [12, 46, 70].

In addition, patients with premature coronary atheromatosis (diagnosed on coronary angiogram or with acute infarction before 45 years) showed elevated serum levels of resistin compared to those without, in the presence of the same risk factors (even in the absence of significant differences between risk factors) [75]. Moreover, in a cohort of patients, resistin has been shown to be an independent predictor of acute coronary syndromes (ACS) (in patients without a history of myocardial infarction or stroke) and for cardiac or cerebrovascular events (in patients with coronary artery disease [63]). Patients with unstable angina and acute myocardial infarction (STEMI or NSTEMI) have elevated serum levels of resistin, suggesting a possible role as a diagnostic biomarker for acute coronary events [76, 77]. Resistin levels increase starting at 3–6 hours after the onset of chest pain and peaked at 12 hours. In an average follow-up of 83.4 months, resistin levels were an independent predictor of a new acute ACS [78, 79] in patients with coronary artery disease [80]. In patients with stable angina treated by PCI, resistin levels increased at 12 hours after the procedure but did not correlate with the increases in troponins levels [81].

The relationship of resistin with the risk of stroke requires a further study. One study on postmenopausal women found an association between increased resistin levels and the risk of stroke, regardless of the presence of obesity and other risk factors for cardiovascular disease [82], a conclusion not confirmed by another large study [83]. Serum resistin levels were able to predict mortality at 5 years and functional outcomes in patients with ischemic stroke [84, 85]. Studies have also identified resistin levels in patients with overt peripheral atherosclerosis. In one study, these were elevated in patients with peripheral arterial disease (PAD) compared to those with ischemic heart disease [86] and plasma levels of resistin and diastolic BP were predictors of new ischemic events and readmission for nonfatal myocardial infarction, heart failure, or critical limb ischemia in these patients. Moreover, in patients with PAD who underwent bypass surgery, increased levels of resistin were able to predict the reduced free survival range without amputation in patients with critical limb ischemia, regardless of the presence of diabetes [87].

3. Conclusion

Nowadays, we are facing a tremendous amount of information that puts us, as practitioners, in front of many decisions that have to be as accurate as possible. Research in the last decades proved that obesity is extremely bound to systemic inflammation and that visceral adiposity increases cardiovascular risk, namely, the risk of cerebrovascular disease and myocardial infarction. Chronic inflammation is linked to endothelial dysfunction and elevated prothrombin activity, and therefore, there is still a need to evaluate the impact of the adipokines in relation to various clinical outcomes of atherosclerosis so that these molecules could become great tools to predict the dynamics and therapeutic response of the disease.

Conflicts of Interest

There is no conflict of interest to be declared.

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Research Article

High-Sensitivity Troponin T and Soluble Form of AXL as Long-Term Prognostic Biomarkers after Heart Transplantation

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Antecedents. Cardiac allograft vasculopathy (CAV) is a frequent complication limiting the long-term (>1 year) survival after heart transplantation (HTx). CAV is initiated by endothelial dysfunction and can lead to severe cardiovascular (CV) complications. Since CAV is often clinically silent, biomarkers could help identifying HTx patients at risk of CAV and their severe complications. *Aim.* Evaluate the clinical yield of high-sensitivity cardiac troponin T (hs-cTnT), marker of cardiomyocyte damage, and the soluble form of AXL (sAXL), biomarker of endothelial dysfunction, to assess the prognosis of long-term cardiovascular (CV) events occurring after HTx. *Methods.* 96 patients were evaluated at least > 1 year after HTx. CAV was evaluated by coronary angiography or multisliced tomography, and hs-cTnT and sAXL measured 6 months before or after CAV evaluation. Patients were followed during 42 ± 15 months for a combined end point including cardiac death, angina or acute myocardial infarction, left ventricular ejection fraction < 50%, or heart failure not due to an acute rejection. *Results.* 51 patients (53%) presented CAV at evaluation; 21 of them had CV events. Hs-cTnT (56 ± 45 versus 20 ± 18 ng/L; $p = 0.04$) and sAXL concentrations (98 ± 51 versus 26 ± 26 ng/L; $p = 0.01$) were significantly higher in patients with CV events. Hs-cTnT (HR 1.03; 95% CI 1.015–1.042, $p = 0.0001$) and sAXL (HR 1.01; 95% CI 1.001–1.019, $p = 0.02$) were independent predictors of CV events. A hs-cTnT concentration < 21 ng/L, detected by AUC ROC, predicted the absence of CV events with a predictive value of 91%; sAXL did not add more predictive value to hs-cTnT. Survival free of CV events was 92% in patients with hs-cTnT < 21 ng/L and 57% in those with hs-cTnT > 21 ng/L ($p < 0.001$). *Conclusion.* Hs-cTnT, but not sAXL, measured during the long-term follow-up of HTx patients appears as a helpful biomarker to identify patients at low risk of adverse CV outcomes.

1. Introduction

Despite the improvement of long-term (>1 year) survival after heart transplantation (HTx), several clinical conditions such as neoplasms, graft failure, infections, and cardiac allograft vasculopathy (CAV) limit it [1, 2]. CAV is the most important cause of cardiovascular (CV) adverse events during follow-up of HTx. CAV is initiated by immunologic and inflammatory phenomena causing endothelial dysfunction and damage. Endothelial damage leads to intimal growth of the coronary epi- and endocardial vessels [3]. CAV is often clinically silent and symptoms can only appear in its advanced stages as acute heart failure (HF) or sudden death. Although CAV has been associated with adverse outcomes, the progression of the disease is variable, making uncertain the prediction of the associated CV events. Some patients may experience a rapid deterioration, while others will remain stable for long time periods [4]. Efforts have been addressed to identify

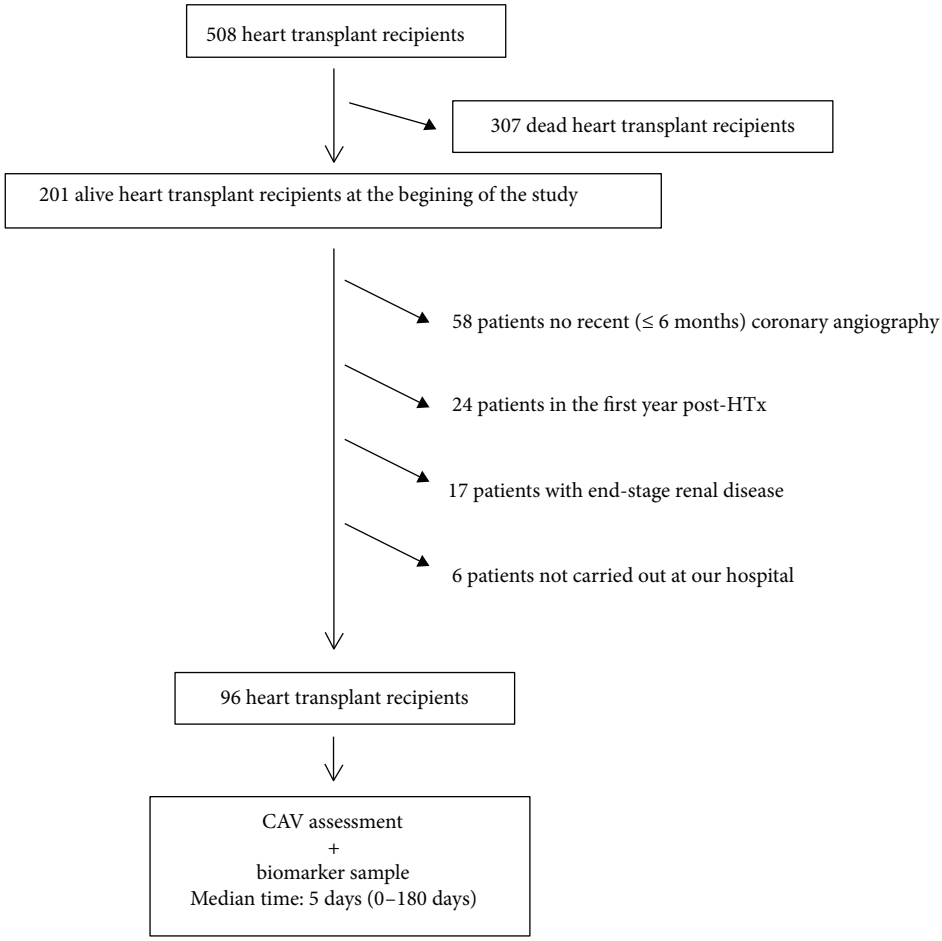


Figure 1: Flowchart of included patients.

biomarkers predicting acute rejection; but results were disappointing [5–10]. Indeed, the role of biomarkers to predict the long-term outcomes of HTx patients is still not well known. Thus, the analysis of recently developed biomarkers linked to the mechanisms involved in CAV development could be worth for improving the risk stratification of HTx patients.

The soluble form of AXL (sAXL), a tyrosine kinase receptor, is considered a biomarker of endothelial dysfunction and associated with myocardial ischemia and heart failure [11]. Cardiac troponins, when measured with high-sensitivity methods (hs-cTn), are not only specific biomarkers of myocardial damage [12] but also sensitive indicators of minor grades of such damage [13]. Both low-grade myocardial necrosis and endothelial dysfunction typically occur in HTx patients developing CAV. The aim of the present study was to evaluate hs-cTnT and sAXL as prognostic biomarkers of long-term (>1 year) adverse outcomes after HTx.

2. Material and Methods

2.1. Study Design. Since the year 1984, our center performed 508 HTx; of them, 201 were alive at the beginning of this study and 96 fulfilled the inclusion criteria, that is, >1 year after the HTx, CAV assessment in the 6 months before/after blood was obtained for biomarker evaluation, an estimated glomerular filtration rate (eGFR) ≥30 mL/min/1.73 m^2 and a follow-up in our center. Of the 105 excluded patients, 58 had no recent (±6 months) CAV assessment, 24 were in the first year post-HTx, 17 had eGFR < 30 mL/min/1.73 m^2, and 6 were followed outside our hospital (Figure 1). All patients included in the study gave written informed consent to participate. The study was approved by our internal review board.

2.2. Clinical Variables. Donor and recipient ages at the HTx time, recipient CV risk factors, etiology (ischemic or nonischemic) of HF leading to HTx, total ischemic time during HTx, acute rejection episodes (first year after HTx), and cytomegalovirus (CMV) infection evaluated monthly during the first year post-HTx, immunosuppressive therapy, and renal function were registered. CMV infection was managed by a preemptive strategy or with prophylactic therapy. Acute rejection was assessed by endomyocardial biopsy (EMB) and was defined according to the International Society for Heart and Lung Transplantation grading system for acute cardiac allograft rejection [14]. Rejection score was defined as the ratio of EMBs with rejection grade > 2R to total EMBs during the first year of follow-up after HTx [15]. CV events were registered during the follow-up as a combined clinical endpoint including cardiac death, acute myocardial infarction or angina, left ventricular ejection fraction (LVEF) <50%, or

Table 1: Demographic and clinical characteristics and biomarkers in 96 HTx patients subdivided according to occurrence or absence of cardiovascular events.

Variables	All $N = 96$	No events $n = 75$	Events $n = 21$	No events versus events P
Recipient age at HTx (years)	47 ± 16	49 ± 15	44 ± 16	0.19
Donor age (years)	40 ± 15	39 ± 13	42 ± 15	0.4
Total ischemic time (min)	183 ± 54	186 ± 52	168 ± 61	0.1
Score rejection (%)	12 ± 14	13 ± 17	12 ± 13	0.9
Cytomegalovirus infection (%)	35 (35%)	29 (39%)	6 (30%)	0.6
Plasma creatinine, umol/L	125 ± 106	104 ± 44	159 ± 120	0.002
Cardiovascular risk factors				
Pre-HTx hyperlipemia (%)	28 (29%)	18 (26%)	10 (55%)	0.02
Post-HTx hypertension (%)	53 (55%)	39 (55%)	14 (78%)	0.7
Post-HTx diabetes (%)	27 (28%)	22 (31%)	6 (33%)	0.1
CAV	51 (53%)	30 (40%)	21 (100%)	
Hs-cTnT (ng/L)	25 ± 27	20 ± 18	56 ± 45	0.04
sAXL (ng/L)	72 ± 35	26 ± 26	98 ± 51	0.01

HTx: heart transplantation; CAV: cardiac allograft vasculopathy; hs-cTnT: cardiac troponin T measured with methods of high sensitivity; sAXL: soluble form of the AXL receptor.

HF not due to an acute rejection. All included patients were followed up until December 2016.

According to the HTx protocol of our center, screening for CAV was based on serial coronary angiography at 1 month, 1 year, 2 years, every 5 years, and whenever it was clinically indicated. Since the year 2012, coronary multislice computed tomography (MSCT) was also introduced after the first year post-HTx, based on its reported accuracy for CAV detection and good correlation with coronary angiography findings [16, 17]. The coronary angiograms and MSCT images were graded from 0 to 3 according to ISHLT consensus [18]. Patients were classified as non-CAV or CAV grade 1, 2, 3, that is, mild, moderate, or severe CAV, respectively.

2.3. Biomarkers. Biomarkers were measured on blood drawn on the same day, but previously to the procedure, or at the time of regular biochemical assessment conducted either 6 months before or after coronary angiography/MSCT. In fact, venous blood samples (lithium heparin) were obtained one month after or before coronary angiography/MSCT in more than half of the evaluated patients. Median time from blood drawing and CAV assessment was 5 days (range from 0 to 180 days). Blood was centrifuged, aliquoted, and stored frozen at −80°C until assayed. Plasma concentrations of sAXL were measured by an enzyme-linked immunosorbent assay (ELISA) previously described [19]; final concentrations were obtained from two replicates of each sample. The mean within- and between-assay imprecision (as coefficient of variation, %CV) was 6.45 and 9.21, respectively. Hs-cTnT was analyzed using an electrochemoluminometric assay in a Cobas e601 platform (Roche-Diagnostics, Basel, Switzerland). The within- and between-assay imprecision at the mean concentration observed and the instrument used was 1.2% and 2.9%, respectively, as %CV.

2.4. Statistical Analysis. Continuous variables are expressed as the mean ± standard deviation (SD) or as median (interquartile range) and differences by Student's t- or Mann-Whitney U tests whenever appropriate. Categorical variables are presented as frequency and percentage. Differences in the categorical variables were assessed by the χ^2 test or Fisher's exact test. Hs-cTnT, sAXL, and plasma creatinine values as variables with a p value < 0.1 and recipient age as a clinical meaningful variable were included in the multivariate models; a backward elimination method was used to identify independent predictors of CV events. A logistic regression model was built to evaluate variables associated to CV events and the value best predicting CV events was obtained from the area under curve ROC (AUC ROC) analysis.

Factors predicting CV events were assessed by Cox multivariate regression; the model included only the main effects of the predictors, without any interaction term or treatment (due to assignment bias in an observational study design). The proportional hazard assumption was evaluated by the Schoenfeld residuals test. The discriminative ability of the model was assessed by the C-statistic. The best cut-off values were identified by AUC ROC, and Kaplan-Meier survival plot was generated for the CV events with comparison by the log-rank test.

The internal validity of the final predictive models was tested for 500 bootstrap resamples, using the "rms" package in the R Project for Statistical Computing. Missing data were imputed using the "mice" package in R (Multivariate Imputation by Chained Equations) whenever necessary ($n = 5$) [20, 21]. A two-sided $p < 0.05$ was considered statistically significant. Data were analyzed with the statistical packages SPSS 24 and R 3.2.

3. Results

Table 1 summarizes the main demographic and clinical characteristics of HTx subjects subdivided according to the event

Table 2: Multivariate analysis for predictors of CV events in HTx patients.

Variables	Association with events				
	HR	95% CI	p	C-statistic	Corrected C-statistic
				0.855	0.836
hs-cTnT	1.03	1.015–1.042	0.0001		
sAXL	1.01	1.001–1.019	0.02		
Recipient age at HTx	0.97	0.941–0.999	<0.05		

HR: hazard ratio; CI: confidence interval; hs-cTnT: cardiac troponin T measured with high-sensitivity methods; sAXL: soluble form of the AXL receptor; HTx: heart transplantation.

occurrence or absence. Mean age was 47 ± 16 years, 78% were men, and the average time from HTx was 9 ± 7 years. Not surprisingly, 79% of the patients had a plasma creatinine ≤ 133 umol/L (1.5 mg/dL), because advanced renal failure was an exclusion criterion for coronary angiography and study inclusion. There were no significant differences between patients with and without events in the recipient and donor age, total ischemia time, score rejection, and rate of CMV infection. Patients were mainly immunosuppressed with combined therapies, particularly including tacrolimus, mycophenolate mofetil, and steroids, and in some cases, cyclosporine or azathioprine; 95% of the patients were receiving statins.

Fifty-one patients (53%) presented CAV and of grade 1 in 27, grade 2 in 5, and grade 3 in 19. Patients with CAV were younger and had higher acute rejection scores and plasma creatinine levels than patients without CAV. There were no significant differences between the two groups in donor's age, total ischemic time, rate of CMV infection, or CV risk factors.

During the mean follow-up time of 42 ± 15 months, 21 patients presented CV events. All patients with CV events had CAV: 16 had CAV grade 3, 1 grade 2 and 4 grade 1. Six patients died of cardiovascular causes (4 CAV grade 3, 1 grade 2, and 1 grade 1), 6 had angina or AMI (5 CAV grade 3 and 1 grade 1), 9 had HF: 6 with LVEF ≤ 50% (all CAV grade 3) without acute rejection and the remaining 3 patients HF with LVEF > 50% (1 CAV grade 3 and 2 grade 1 with a severe restrictive pattern).

There was any significant correlation between biomarker concentrations and time after HTx (rho Spearman: sAXL = 0.123, $p = 0.23$; hs-cTnT = 0.168, $p = 0.1$). There existed significant differences between patients with and without CV events in hs-cTnT (56 ± 45 versus 20 ± 18 ng/L; $p = 0.04$) and sAXL concentrations (98 ± 51 versus 26 ± 26 ng/L; $p = 0.01$) (Table 1). After adjusting for clinical variables, multivariate analysis identified hs-cTnT, sAXL, and younger recipient age as independent predictors of CV events (Table 2). The addition of sAXL to hs-cTnT values and recipient age only increased the discrimination capacity to predict events by 7.3%, but the improvement was not significant $p < 0.10$. The AUC ROC analysis identified that a hs-cTnT concentration ≥ 21 ng/L had positive predictive value of CV events of only 43%, but a concentration < 21 ng/L had a negative predictive value of 91%. The best cut-off concentration for sAXL with similar negative predictive value than that of hs-cTnT was 66.5 ng/L (Figure 2). However, using this cutoff value, the positive predictive value for event prediction was lower (31%) than that of hs-cTnT.

Survival free of CV events for a hs-cTnT < 21 ng/L was 92% compared with 57% in patients with hs-cTnT ≥ 21 ng/L ($p < 0.001$) (Figure 3).

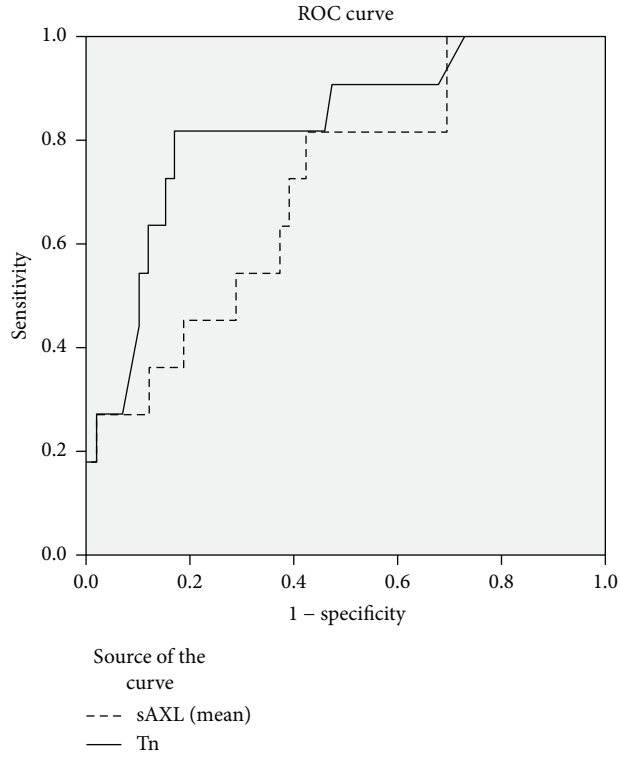
4. Discussion

The current study analyzed the prognostic value of two biomarkers of cardiomyocyte lesion and endothelial dysfunction, hs-cTnT and sAXL, respectively, to predict severe CV events in long-term surviving patients (42 ± 15 months) after HTx. We found that plasma concentrations of hs-cTnT, but not those of sAXL, are strong negative predictors of the probability of suffering long-term CV events.

CAV is one of the more frequent and severe causes of CV complications in HTx patients. In the study, all patients with CV events in the follow-up have different CAV degrees, but most have the most severe form of the disease (CAV degree 3). CAV begins with an endothelial dysfunction and lesion. Consequently, increased values of sAXL, an endothelial dysfunction biomarker, should be expected in patients with CAV. We found increased sAXL concentrations in those HTx patients with CV events, but the biomarker did not add prognostic value of CV events to concentrations of hs-cTnT when analyzed in multivariable analysis.

Cardiac troponins (cTn) are specific biomarkers of cardiomyocyte injury and have become the gold standard for diagnosing myocardial lesions or infarctions [12, 13]. The new, high-sensitivity assays (Hs-cTn) allow to measure very small concentrations of cTn and distinguish between values found in healthy subjects and in subjects with subtle cardiac damage as the produced by coronary ischemia. In contrast, sAXL protein is expressed in many organs and tissues; its concentration in skeletal muscle is twice than that of cardiac tissue, and it is also expressed at higher concentrations than in heart tissue in the small bowel, colon, lungs, kidneys, and pancreas, among others [22]. Moreover, the methods developed to measure sAXL (ELISA) are not equally sensitive as the methods developed to measure hs-cTn. All these reasons can explain why circulating sAXL concentrations cannot sensitively and accurately predict the CV complications associated to CAV in our HTx population.

Small increases of hs-cTn have been associated with worse prognosis in patients with non-ST elevation acute coronary syndromes and heart failure [23]. Given that cTnT was the first cTn measurable with high-sensitivity methods



Diagonal segments are produced by ties.

Area under the curve

Test result variable (s)	Area	Std. Error	Asymptotic sig.	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
sAXL (mean)	0.710	0.082	0.028	0.551	0.870
Tn	0.827	0.069	0.001	0.692	0.963

Figure 2: ROC curves and its p value for hs-cTnT and sAXL.

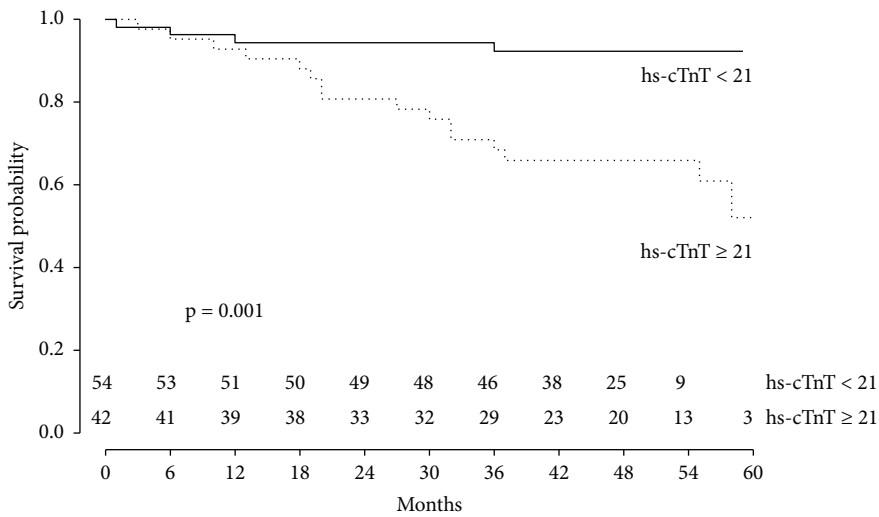


Figure 3: Kaplan-Meier survival curve for heart-transplanted patients according to the hs-cTnT value obtained > 1 year after transplantation and close to CAV evaluation. Comparison of survival free of cardiovascular events for recipients with hs-cTnT <21 ng/L or hs-cTnT ≥ 21 ng/L.

(hs-cTnT), there exists much more scientific evidence for this biomarker than for others similar like hs-cTnI. Despite being accurately used in the diagnosis of myocardial lesions and infarction, hs-cTnT has been poorly studied as a marker of rejection and CV events after HTx. Routine screening and monitoring of CAV is based on serial coronary angiography

or multislice computed tomography (MSCT), both exposed to morbidities related to iodine contrast infusion and X-ray exposure. Furthermore, coronary angiography is an invasive technique which cannot be performed to patients with advanced deterioration of kidney function. In a previous study, hs-cTnT concentrations at 6 weeks after HTx were independent predictors of 12-month mortality [24]. CAV can be responsible for high hs-cTnT values; but conversely, normal hs-cTnT could be predictive of CAV absence and its complications and help to identify those HTx patients at low risk of CV events to avoid an excess of coronariographies or MSCT. Recently, the high negative predictive value of low plasma hs-cTn concentrations to rule out acute HTx rejection has been described by our group and others [25, 26]. In our study, hs-cTnT concentrations < 21 ng/L showed a high negative predictive value (91%) of CV events and were associated with a 92% event-free survival in the follow-up. Based on these results, it is likely that HTx recipients with low hs-cTnT values (<21 ng/L) could undergo less frequent CAV evaluation. Recently, the AlloMap gene expression profile test has also shown a high negative predictive value to estimate the likelihood of events in patients beyond 315 days post-HTx [27]. However, this test is expensive, not widespread used, and only limited data supports its use for prognosis assessment; in contrast, hs-cTnT is an inexpensive test that can be processed in most centers and can be applied to all HTx patients regardless their renal function status.

In conclusion, based on the high negative predictive value observed in our study, the cut-off value of hs-TnT < 21 ng/L may offer a useful means to help identifying HTx patients at low risk of CV events and therefore better prognosis reducing the requirement of the more expensive and harmful image techniques.

4.1. Study Limitations. The relatively small size of the study combined with the fact that only 6 patients died during the follow-up precluded assessing the values of hs-cTnT to predict crude mortality.

The study included those HTx patients surviving to the intervention for >1 year and excluded the survivors with advanced renal failure. This could contribute to select those HTx survivors with a good health status. Thus, results found apply to this subgroup of HTx patients. However, this "bias selection" allows us to analyze a population on which the hs-cTnT concentration was mainly influenced by cardiac status and not by confounding factors like renal function.

Finally, although coronary angiography is the gold standard, multislice computed tomography (MSCT) has also been validated for CAV assessment [16, 17]. Since advanced renal failure was an exclusion criterion for coronary angiography, we cannot exclude advanced renal failure as an additional marker for poor survival.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Procalcitonin (PCT) Predicts Worse Outcome in Patients with Chronic Heart Failure with Reduced Ejection Fraction (HFrEF)

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Introduction. Procalcitonin (PCT) is an excellent marker of sepsis but was not extensively studied in cardiology. The present study investigated PCT plasma concentration in patients with chronic heart failure with reduced ejection fraction (HFrEF) and its prognostic value during 24-month follow-up. *Material and Methods*. Study group consisted of 130 patients with HFrEF (LVEF ≤ 45%) and 32 controls. PCT level was assessed on admission in all patients. Telephone follow-up was performed every three months over a period of 2 years. Endpoints were death of all causes and readmission for HFrEF exacerbation. *Results*. HFrEF patients had significantly higher PCT concentration than controls (166.95 versus 22.15 pg/ml; $p < 0.001$). Individuals with peripheral oedema had increased PCT comparing to those without oedema (217.07 versus 152.12 pg/ml; $p < 0.02$). In ROC analysis, PCT turned out to be a valuable diagnostic marker of HFrEF (AUC 0.91; $p < 0.001$). Kaplan-Meier survival curves revealed that patients with PCT in the 4th quartile had significantly lower probability of survival than those with PCT in the 1st and 2nd quartiles. In univariate, but not multivariate, analysis, procalcitonin turned out to be a significant predictor of death during 24-month follow-up. (HR 1.002; 95% CI 1.000–1.003; $p < 0.03$). *Conclusions*. Elevated PCT concentration may serve as another predictor of worse outcome in patients with HFrEF.

1. Introduction

Growing population of patients with chronic heart failure is a major challenge for healthcare systems throughout the world. High rate of expensive rehospitalization is one of the main causes of economic burden associated with CHF. Risk stratification leading to identification of subgroups with the worst outcome could make the treatment process more efficient with the most aggressive strategies reserved for those with gloomy prognosis. Search for diagnostic and prognostic markers of CHF led to the discovery of the immense clinical role of human natriuretic peptides [1–3]. Haemodynamic changes occurring in failing heart resulting in natriuretic peptide synthesis are not the only one alteration in this multifaceted disease. Immunologic and inflammatory mechanisms are also affected during the natural course of chronic heart failure [4, 5]. Clinical utility of established inflammatory markers such as C-reactive protein, interleukins, and tumour necrosis factor has been extensively studied in CHF population [6–8]. Calcitonin-derived propeptide—procalcitonin—was identified in the 1990s as a marker of sepsis and invasive bacterial infection with excellent specificity and sensitivity [9]. Increased concentration of procalcitonin was reported not only in infectious diseases but also in ischemic stroke [10], in lupus exacerbation [11], and in patients with medullary thyroid carcinoma [12]. Its plausible role in cardiovascular disorders was not thoroughly investigated. Reports on procalcitonin level alterations in heart diseases are scarce and far between and are mainly focused on open-heart surgery complications. Procalcitonin is also a valuable tool in differentiating patients presenting with acute dyspnoea into those with acute heart failure and those with pneumonia [13].

Table 1: Clinical and laboratory characteristics of stable and exacerbated patients [14].

	All	Stable	Exacerbated	p
		Clinical and demographic indices		
n	130	65	65	—
Males	107	55	52	ns
Age (years; mean ± SD)	59.8 ± 13.1	57.3 ± 9.8	61.5 ± 14.1	ns
BMI (kg/m^2; mean ± SD)	28.7 ± 5.8	28.2 ± 5.6	28.6 ± 6.0	ns
Ischemic aetiology	66 (53%)	32 (49%)	34 (56%)	ns
NYHA II/III/IV	39%/51%/9% 52/66/12	55%/45%/0% 36/29/0	24%/57%/18% 16/37/12	<0.001*
Peripheral oedema	40 (31%)	8 (12%)	32 (49%)	<0.05*
Pulmonary congestion	37 (28%)	5 (8%)	32 (49%)	<0.05*
LVEF (%; mean ± SD)	27 ± 8	27 ± 7	26 ± 8	ns
DM	47 (36%)	21 (32%)	26 (40%)	ns
Smoking	37 (28)	19 (25%)	18 (24%)	ns
Death	32 (25%)	12 (18%)	20 (30%)	<0.001*
Hospitalization	60 (46%)	33 (51%)	27 (41%)	ns
		Laboratory tests		
Hb [g/dl]	13.9 ± 1.5	14.1 ± 0.9	13.7 ± 1.9	ns
Nt-proBNP [pg/ml]	1862 ± 5957	1643.92 ± 1776	5142 ± 7391	<0.0001*
Troponin T [μg/l]	0.02 ± 0.03	0.03 ± 0.03	0.03 ± 0.02	ns
		Treatment		
ACEI	105 (82%)	51 (78%)	54 (83)	ns
ARB	23 (18%)	14 (22%)	9 (14%)	ns
Betablockers	129 (99%)	65 (100%)	64 (99%)	ns
Statin	105 (81%)	58 (89%)	47 (72%)	ns
ASA	61 (47%)	26 (40%)	35 (54%)	ns
Digoxin	35 (27%)	22 (34%)	13 (20%)	<0.05*
Spironolactone	96 (74%)	45 (69%)	51 (78%)	ns
Eplerenone	23 (18%)	15 (23%)	8 (12%)	<0.05*
Diuretic	101 (78%)	36 (55%)	65 (100%)	<0.05*

BMI: body mass index; NYHA: New York Heart Association; LVEF: left ventricular ejection fraction; DM: diabetes mellitus; Hb: haemoglobin; NT-proBNP: N-terminal probrain natriuretic peptide; ACEI: angiotensin converting enzyme inhibitors; ARB: angiotensin receptor blockers; ASA: acetylsalicylic acid. *p value for the difference between stable and exacerbated patients.

Taking into account the diagnostic and prognostic significance of the traditional inflammatory markers, we decided to evaluate procalcitonin plasma level in patients with chronic heart failure with reduced ejection fraction and assess its impact on prognosis in this population.

1.1. Aim of the Study. The aim of the study was to investigate plasma concentration of procalcitonin in patients with chronic heart failure with reduced ejection fraction and assess its prognostic value in this population during 24-month follow-up.

2. Material and Methods

The study group consisted of 130 consecutive patients admitted to the university hospital department of cardiology with chronic heart failure with reduced ejection fraction. Half of them had sign or symptoms of heart failure exacerbation. The other 50% of patients were admitted electively for device implantation or periodic assessment of the disease progression. The inclusion criteria were as follows: chronic heart failure with left ventricular ejection fraction ≤ 45% assessed within past 6 months. Patients with acute coronary syndrome, acute heart failure, active infection or cancer, fever, known or suspected infection, chronic obstructive pulmonary disease, chronic inflammatory diseases, decompensated diabetes, and advanced renal insufficiency with glomerular filtration rate below 30 ml/min were excluded from the study. Clinical characteristics of the study group are summarized in Table 1.

The control group consisted of 32 healthy adult volunteers matched for age and sex. They were recruited from the outpatient cardiology department after the exclusion of cardiovascular diseases and other condition outlined in the exclusion criteria.

After explanation of the study design, each study participant signed an informed consent. Study protocol was in compliance with the principles of the Declaration of Helsinki and

Table 2: Comparison of white blood cell count (WBC), procalcitonin (PCT), and high sensitivity C-reactive protein (hsCRP) plasma concentration in patients with chronic heart failure: stable and exacerbated and in controls.

Group	Minimum	Lower quartile	Median	Upper quartile	Maximum	p
			Procalcitonin [pg/ml]			
Control	1.19	13.00	22.15	48.74	200	
CHF all	14.71	112.27	166.95	284.53	927	<0.0001*
CHF stable	14.71	118.65	163.71	280.47	800.24	
CHF exacerbation	22.83	105.46	175.19	286.54	927.00	
			hsCRP [mg/l]			
Control	0.3	1.7	2.2	2.5	6.6	
CHF all	0.6	1.9	4.0	7.8	48	<0.01*
CHF stable	0.6	1.6	3.4	7.2	23.7	
CHF exacerbation	0.8	2.1	4.6	8.2	48	
			WBC [G/l]			
Control	4.61	5.70	6.27	8.87	9.18	
CHF all	4.03	5.98	7.32	11.12	13.74	ns
CHF stable	4.83	6.10	7.28	11.23	13.74	
CHF exacerbation	4.03	5.65	7.37	10.91	12.82	

*Difference between CHF all and controls.

was approved by the Bioethical Committee of the Collegium Medicum of Nicolaus Copernicus University.

At baseline routine, laboratory tests including complete blood count, blood electrolytes, creatinine, troponin T (TnT), N-terminal brain natriuretic propeptide (NT-proBNP), and C-reactive protein (CRP) concentration were performed in each study participant. Blood samples for determination of PCT plasma concentration were collected in the supine position into Vacutainer® test tubes containing anticoagulant—3.2% sodium citrate. The blood samples were then centrifuged at 3000 ×g at 4°C for 10 minutes, and the obtained plasma was sampled into eppendorf test tubes and frozen at −80°C for no longer than 3 months before the measurement was taken. Controls' plasma samples were prepared in the same way as from CHF patients. Freeze-thaw cycles were avoided before analysis.

The remaining tests were performed in the hospital laboratory. NT-proBNP plasma level was determined by means of sandwich chemiluminescence immunoassay (Elecsys® proBNP II, Roche Diagnostics, Germany), and C-reactive protein concentration was measured with the use of high-sensitivity immunoturbidimetric method (CRP OSR6199 Highly Sensitive Application, Olympus).

PCT plasma concentration was determined in the university Department of Immunology with the use of commercially available, highly sensitive enzyme-linked immunosorbent assay (SEA 698Hu, USCN Life Science,) according to the manufacturer instructions. The intra-assay and interassay coefficients of variation were 8%–9.5% and 7.5%–9%, respectively.

All-cause mortality and hospitalization for the exacerbation of heart failure were the study endpoints. Telephone follow-up visits were performed every three months for 2 years.

2.1. Statistical Analysis. Statistical analyses were performed with the use of the PQStat 1.4.8 software. Kruskal-Wallis test with Bonferroni correction was used to determine the difference of the studied parameters between controls and stable and exacerbated CHF patients. Mann–Whitney U test was used to analyse differences in PCT plasma level between patients with or without signs of hypervolaemia. Prognostic value of PCT plasma concentration was analysed with logistic regression models. Receiver operator curves (ROC) were used to assess diagnostic significance of PCT. Kaplan-Meier survival curves were performed to explore the impact of PCT on all-cause mortality. A probability less than 0.05 was considered statistically significant.

3. Results

Baseline study and control group characteristic is presented in Table 1. Half of patients were admitted to the hospital with exacerbation, and half were in stable condition. Although there is no precise definition of heart failure exacerbation, we decided to include only these patients who demonstrated certain dynamics of increasing hypervolaemia ultimately urging patient to search for medical care. Low-output states were excluded from the study as in our opinion in initial stages it is difficult to make a clear distinction between plain HF exacerbation with hypotension and developing cardiogenic shock being a classical form of acute heart failure.

Plasma procalcitonin concentration was significantly higher in CHF patients than in controls. (166.95 versus 22.15 pg/ml; $p < 0.001$). Yet, no difference in PCT was recorded between exacerbated and stable study participants. There was statistically significant correlation between hsCRP level and PCT concentration (r 0.16; $p < 0.05$); however, no correlation between white blood cell count and PCT level was observed. PCT values together with other common inflammatory markers are presented across study groups in Table 2.

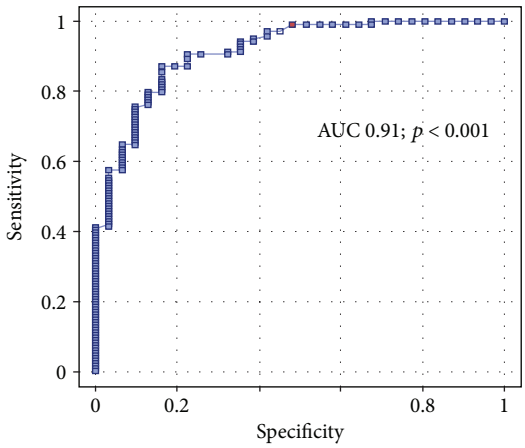


Figure 1: Receiver-operator curve of procalcitonin diagnostic value.

ROC analysis showed that procalcitonin is a valuable marker of chronic heart failure with reduced ejection fraction. (AUC 0.91; $p < 0.001$; Figure 1).

Moreover, PCT was also increased in individuals with peripheral oedema comparing to patients without oedema on physical examination performed on admission (217.07 versus 152.12 pg/ml; $p < 0.02$). In contrast, another sign of hypervolaemia—pulmonary congestion—did not influence PCT concentration (Table 3).

In univariate analysis, procalcitonin turned out to be a significant predictor of death during 24-month follow-up (HR 1.002; 95% CI 1.000-1.003; $p < 0.03$). Kaplan-Meier survival curve highlights significant survival difference between patients with procalcitonin concentration in the 1st or 2nd and 4th quartile ($p < 0.002$) (Figure 2).

In multivariate model, only NT-proBNP (HR 1.036; 95% CI 1.008-1.0117; $p < 0.02$) and age (HR 1.061; 95% CI 1.007-1071; $p < 0.03$) independently predicted mortality. PCT plasma concentration did not allow for the prediction of the second prespecified endpoint—readmission for heart failure exacerbation.

There were statistically significant correlation between PCT and NYHA (r 0.3; $p < 0.0003$), NT-proBNP (r 0.2; $p < 0.01$) concentration, and negative correlation between PCT and LVEF ($r - 0.17$; $p < 0.04$).

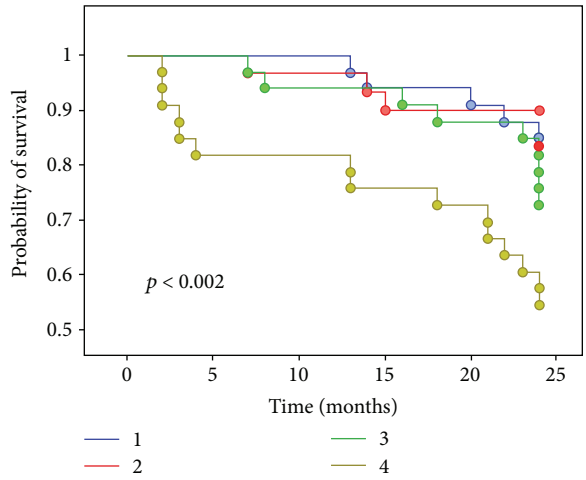
4. Discussion

Similarly to our findings, Canbay et al. in a retrospective case-control study observed increased serum procalcitonin concentration in patients with chronic heart failure with LVEF < 45% comparing to controls. Moreover, individuals hospitalized with CHF had higher PCT than those in an outpatient clinic. A cut-off value set at 0.09 ng/ml (90 pg/ml) PCT allows for differentiating patients with CHF from those without the disease with all controls having negative PCT [15]. According to ROC analysis that we used to assess diagnostic accuracy of PCT, lower cut-off value of 22.8 pg/ml (99% sensitivity and 49% specificity) is optimal for diagnosing CHF. Half of our study group consisted of patients admitted to the hospital with signs of exacerbation; however, we did not observe any more pronounced elevation of procalcitonin concentration in this subgroup. After physical examination performed on admission, we also divided all patients according to signs of hypervolaemia, and those with peripheral oedema turned out to have significantly higher concentration of PCT comparing to those without oedema, but this was not the case in individuals with pulmonary congestion only. Plausible explanation of this interesting phenomenon comes from the study by Mollar et al. Authors assessed procalcitonin level together with multiple other immunologic and inflammatory markers in 261 patients diagnosed with the episode of acute heart failure after exclusion of active infection in an emergency department setting. Significant correlation was found between PCT concentration and surrogate markers of inflammation including leukocyte count, low lymphocyte number, low high-density lipoproteins, and endotoxin level. Moreover, signs of venous congestion such as sodium and gamma-glutamyl transpeptidase level and low estimated glomerular filtration rate turned out to increase PCT concentration. Authors conclude that all the evidence points out toward the endotoxin stimulation resulting from bacterial translocation through the intestine wall in patients with peripheral congestion. Altered intestine function leading to the stimulation of inflammatory process in patients with heart failure is not a new concept in pathophysiology of this multifactorial syndrome [16]. The most recent publication by Pasini et al. proves that not only intestine permeability is increased in CHF patients but also massive bacterial and fungal overgrowth can be observed in this population. Apart from microbiologic investigation and intestinal barrier properties evaluation, Pasini et al. assessed also right atrial pressure (RAP) as a surrogate marker of congestion and C-reactive protein as the most common marker of systemic inflammation. The observed positive correlation between increased intestine permeability and RAP is in concordance with our findings of higher procalcitonin level in patients with peripheral oedema [17]. All together, these findings provide plausible mechanistic explanation for the observed CHF-associated increase of procalcitonin. Yet, we did not find any difference in terms of CRP concentration between patients with or without peripheral oedema. In our opinion, this observation indicates that PCT with its high sensitivity and specificity in diagnosis of bacterial bloodstream infection may be a more valuable indicator of bacterial translocation associated with advanced heart failure than such a versatile marker as CRP. Another observation confirming that heart failure may be a reason behind PCT elevation comes from a relatively recent study by Wang et al. Authors in a cohort of 4698 patients with dyspnoea recorded higher PCT concentration in patients with heart failure than in controls together with higher PCT in CHF complicated by the infection comparing to patients with infection only. These results indicate the significant diagnostic value of PCT in differential diagnosis of patients with dyspnoea managed in an emergency setting [18].

In our study group, PCT, in univariable model, turned out to significantly impact all-cause mortality during 24-month observation. Patients with PCT in the 4th quartile had significantly lower chance of survival than those in the

Table 3: Procalcitonin in patients with or without pulmonary congestion and peripheral oedema.

Group	Minimum	Lower quartile	Median	Upper quartile	Maximum	Mann–Whitney U test
			Procalcitonin [pg/ml]			
Oedema	22.83	146.4	217.07	355.91	787.36	$p = 0.02$
No oedema	14.71	99.5	152.12	246.76	927.00	
Congestion	22.83	122.4	207.47	356.88	927.00	$p = 0.07$
No congestion	15.71	108.18	153.56	234.32	787.36	



No at risk						
PCT in 1st quartile	33	33	33	31	31	28
PCT in 2nd quartile	32	32	31	31	29	29
PCT in 3rd quartile	32	32	30	30	28	23
PCT in 4th quartile	32	26	26	24	23	18

Figure 2: Kaplan-Meier survival curves for quartiles of PCT concentration.

1st and 2nd quartiles. Prognostic value of procalcitonin concentration has been described previously, however, only in patients with acute heart failure (AHF). Loncar et al. evaluated the impact of both baseline procalcitonin level and the increase of PCT concentration during hospitalization in patients admitted with AHF episode. Persistent elevation of PCT as well as its increase during the first 72 hours of hospitalization was associated with the worst outcome during 3-month follow-up [19]. Similar findings come from BACH trial in which PCT turned out to predict worse outcome not only in patients with pneumonia but also in individuals with acute heart failure. BACH investigators underscore that PCT is not only a valuable addition to routine procedures used in initial diagnosis in patients presenting with dyspnoea but also provides prognostic information and can help to choose treatment modality [13]. The most recent study addressing PCT's role in predicting outcome in patients with heart failure also focuses on cases of acute heart failure. Villanueva et al. followed 261 consecutive patients admitted with the diagnosis of AHF without signs of infection in whom baseline PCT was determined. PCT accurately predicted both death of all causes and rehospitalization. In our research, we did not record the influence of PCT concentration on readmission; however, the length of observation—considerably shorter in our study—might be the reason behind the lack of the relationship between PCT and this endpoint [20].

5. Conclusions

The present study is to the best of our knowledge the first research on procalcitonin diagnostic and prognostic value in chronic heart failure population. Following promising results of trials involving patients with AHF, this study provides another set of evidence on the emerging role of procalcitonin in heart failure diagnostic process and risk stratification. Since the incorporation of natriuretic peptide assessment into routine clinical practice, numerous plausible markers of heart failure have been tested with varying results. Taking into account the high residual risk reflecting multifaceted pathophysiology of heart failure, natriuretic peptides even with their immense clinical role seem to be insufficient. Thus, multimarker approach may help to refine therapeutic strategies and allow for tailored treatment adjusted to the individual clinical and biochemical profile of each heart failure patient.

Data Availability

All data used to support the finding of this study are available from the corresponding author upon request.

Conflicts of Interest

Authors declare no conflict of interest.

Acknowledgments

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Research Article

A Preliminary Study of microRNA-208b after Acute Myocardial Infarction: Impact on 6-Month Survival

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Introduction. miRNAs contribute to a variety of essential biological processes including development, proliferation, differentiation, and apoptosis. Circulating microRNAs are very stable and have shown potential as biomarkers of cardiovascular disease. microRNA-208b expression was increased in the blood of patients with acute myocardial infarction (AMI) and has been proposed as a biomarker for early diagnosis. In this pilot study, we investigate the potential of circulating miR-208b as a prognostic biomarker of 6-month survival in AMI patients. *Methods*. Plasma samples from 21 patients and 8 age- and gender-matched healthy adults were collected, and circulating levels of miR-208b were detected using quantitative real-time PCR. *Results*. miR-208b levels were higher in healthy control subjects (9.6-fold; $P \leq 0.05$). Within the AMI patients, the levels of miR-208b were significantly lower in the *survivor* versus *nonsurvivor* group (fold change = 6.51 and 14.1, resp.; $P \leq 0.05$). The Kaplan-Meier curve revealed that the 6-month survival time was significantly higher among AMI patients with a relative expression of miR-208b lower than 12.38. The hazard ratio (HR) for the relative expression of miR-208b (<12.38 was the reference) was 5.08 (95% CI: 1.13–22.82; $P = 0.03$). *Conclusion*. Our results showed that elevated miR-208b expression was associated with reduced long-term survival in AMI patients. These pilot data indicate the need for a large follow-up study to confirm whether miR-208b can be used as a predictor of 6-month survival time after AMI.

1. Introduction

Acute myocardial infarction (AMI) occurs as a result of the acute necrosis of myocardial tissue following persistent and severe ischemia [1]. AMI is one of the most common cardiovascular diseases and one of the leading causes of mortality and morbidity across the globe. 17 million people die annually of cardiovascular diseases with 10 million being in developing countries [2–4]. Patients with a comorbid diagnosis of AMI had two to three times the case-fatality rate of patients in whom AMI was a primary diagnosis [5]. It is predicted that cardiovascular diseases will constitute 36% of all deaths globally in 2020 [6]. Some conventional biomarkers, such as blood troponins, cardiac myoglobin, and creatine kinase-MB (CK-MB) are currently used for clinical diagnosis of AMI [7].

microRNAs (miRNAs) are small (19–25 nucleotides in length), noncoding, and highly conserved RNA molecules which are involved in the regulation of gene expression. The regulatory functions of miRNAs are achieved through the RNA-induced silencing complex [8]. miRNAs control a variety of essential biological processes including development, proliferation, differentiation, and apoptosis [9]. Dysregulated tissue expression of miRNAs contributes to various diseases such as cancer and cardiovascular disease [10–12]. Recent studies demonstrated that miRNAs play a crucial role in AMI mechanisms such as atherosclerotic plaque rupture, blood platelet aggregation, and necrosis of heart cells after blockage of the coronary artery [13].

Many miRNAs are remarkably stable and easily detectable in the peripheral blood or plasma [14, 15]. The levels of circulating miRNAs may differ under pathological conditions [16–18]. This suggests plasma miRNA concentrations may be used as superior biomarkers for the diagnosis and prognosis of diseases in humans [19, 20]. The levels of several miRNAs such as miR-1, miR-133a, miR-208b, miR-499, and miR-328 are altered in the blood and plasma during AMI [21–26]. miR-149, miR-499, and miR-208b are increased immediately after percutaneous coronary intervention (PCI) and therefore have promise as diagnostic and prognostic biomarkers in AMI [27]. miR-208 is produced exclusively in the rat myocardium and is considered as a biomarker of myocardial injury in rats [28]. The same study reported that the plasma level of miR-499 may also be a useful biomarker of myocardial infarction in humans [28]. The present study aimed to investigate the potential prognostic value of circulating miR-208b in AMI patients with respect to 6-month survival time.

2. Methods

2.1. Patient Characteristics. This pilot prospective prognostic study recruited AMI patients sequentially referred to the Imam Hossein Hospital affiliated to the Shahid Beheshti University of Medical Sciences between January and December 2016. The study was approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences at Tehran, Iran, and all patients gave informed consent.

Patients with acute ischemic chest pain, abnormal electrocardiogram (pathological Q wave and ST-segment elevation), and increased levels of troponin and creatine kinase greater than 2 times the upper limit of the normal range with a diagnosis of AMI were enrolled into the study. Patients with a previous history of venous thrombolytic injection or receiving anticoagulant, previous MI or PCI, hematological diseases, acute or chronic infection, significant hepatic dysfunction, renal failure, or known or cured malignancy were excluded. The patients were admitted to hospital no more than 12 h after the emergence of symptoms, and blood samples were collected immediately after admission. A cutoff value of 55% was used for the ejection fraction (EF). If the diagnosis of AMI was confirmed, then the blood samples were submitted to the reference laboratory for miRNA analysis.

Five-milliliter venous blood samples of patients with AMI were collected in EDTA anticoagulant tubes at admission. Samples were centrifuged at 3000 ×g for 10 min at 4°C, and then the supernatant was isolated and centrifuged at 12,000 ×g for 10 min at 4°C. Plasma was collected and stored at −80°C until RNA extraction. Moreover, 8 age- and gender-matched healthy volunteers with normal electrocardiograms and no history of cardiovascular diseases were recruited as a control group.

2.2. RNA Extraction and cDNA Synthesis. Serum-free miRNAs in patients and the control group were extracted using an RNA extraction kit (Exiqon, Vedbæk, Denmark). Extracted RNA was reverse transcribed using the miRCURY LNA Universal RT microRNA cDNA Synthesis Kit (Exiqon) according to the manufacturer's instructions.

2.3. Real-Time Quantitative PCR Analysis

2.3.1. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Real-time PCR assays were performed using the ExiLENT SYBR® Green Master Mix Kit (Exiqon). LNA primers were purchased from Exiqon. cDNA was diluted 10x and added to the PCR reactions according to the manufacturer's instructions. The real-time PCR program included the following steps: an initial denaturation step at 95°C for 10 min and 50 cycles of amplification that consisted of a denaturation step (10 s at 95°C) and an annealing step (60 s at 60°C). The expression levels of miR-208b were normalized to the level of miR-16 as control using the efficiency-corrected calculation models of the Pfaffle method [29].

$$\text{Ratio} = \frac{(\text{Etarget})\,\Delta\text{Ct target}(\text{control} - \text{sample})}{(\text{ERef})\,\Delta\text{Ct Ref}(\text{control} - \text{sample})}. \quad (1)$$

2.4. Statistical Analysis. Data were presented using mean (SD) and frequency and 95% confidence interval (95% CI). Independent sample *t*-tests or Mann–Whitney *U* tests and chi-square test were used to investigate the differences in continuous and categorical variables, respectively. The Kaplan-Meier method was used for depicting univariate survival curves illustrating the association between the biomarker expression and disease-specific survival (DSS). DSS

Table 1: Clinical features and risk factors of the cohort.

Characteristics	All ($n = 21$)	Survivor ($n = 14$)	Nonsurvivor ($n = 7$)	P value
Age	62.71 (12.75)	58.57 (11.37)	71 (11.88)	0.15
Sex (M/F)	15/6	9/5	6/1	0.31
Smoking (yes/no)	8/13	5/9	3/4	0.75
Diabetes (yes/no)	4/17	3/11	1/6	0.69
Hypertension (yes/no)	13/8	8/6	5/2	0.52
Hyperlipidemia (yes/no)	10/11	6/8	4/3	0.54
Cardiac troponin T (ng/mL)	10.08 (10.18)	10.54 (10.20)	9.15 (10.89)	0.77
Decrease in EF* (yes/no)	6/13	4/10	2/3	0.64
Relative expression	8.63 (12.83)	4.58 (4.85)	16.72 (19.58)	0.04

*Cutoff = 0.55.

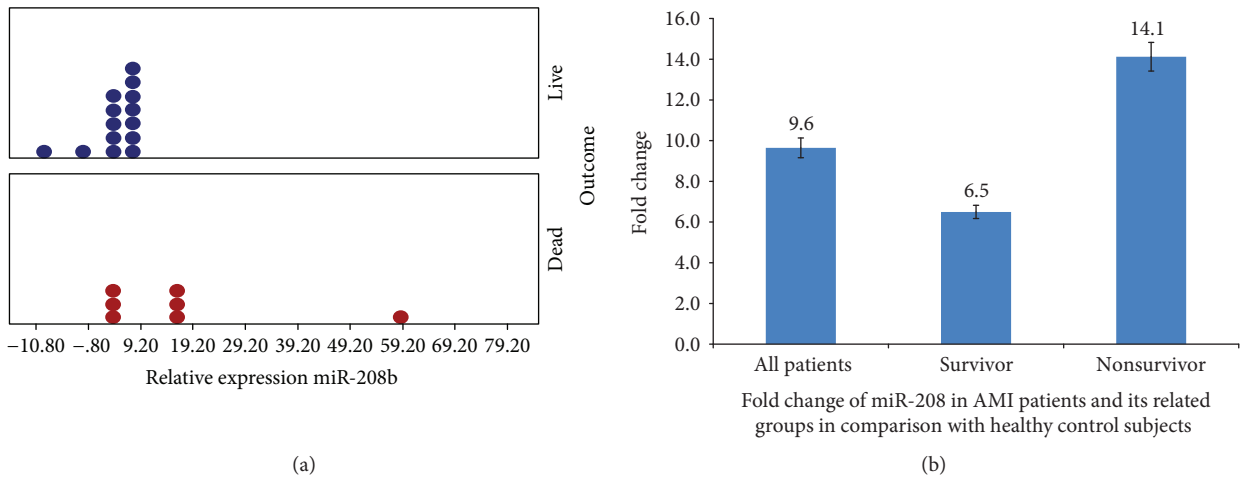

Figure 1: (a) Relative expression of circulating miR-208b in AMI patients in the survivor and nonsurvivor groups ($P = 0.03$). The expression of miR-208b was 9.6-fold higher in the AMI group compared with the healthy control subjects. (b) The relative expression of miR-208b was increased in both survivor and nonsurvivor groups in comparison to healthy subjects (fold change = 6.51 and 14.1, resp.). Plasma samples were collected upon admission no more than 24 h after AMI onset (in all cases, P value ≤ 0.05).

was defined from the date of enrollment until the time of AMI death. Statistical significance between the survival curves was assessed utilizing the log-rank test. The Cox proportional hazard model was used to estimate the hazard ratio of death for miR-208b. The cutoff value was determined based on the Youden index. P values less than 0.05 were considered statistically significant for all analyses. All statistical analyses were performed using the statistical package IBM SPSS, version 21 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Clinical Characteristics of Patients. Among the 21 patients diagnosed with AMI, 7 patients died within six months of diagnosis. Both the nonsurvivor and the survivor groups were predominantly male (6/7 and 9/14, resp.). The demographics of the patients in this study are shown in Table 1. No significant differences were observed in the personal history including hypertension, hyperlipidemia, diabetes, cardiac troponin T, and the left ventricular ejection fraction (EF) and smoking between nonsurvivor and survivor patients.

3.2. Assessment of the Circulating miR-208b Levels. The level of miR-208b was measured in the plasma of 21 AMI patients according to the survival time as well as in the healthy controls. The expression of miR-208b was significantly greater in the AMI group compared with healthy control subjects (fold change = 9.6, $P \leq 0.05$) (Figure 1). As shown in Figure 1, the relative expression of miR-208b was increased in both survivor and nonsurvivor groups as compared to healthy subjects (fold change = 6.51 and 14.1, resp.; $P \leq 0.05$). There was no effect of age on the miR-208b level (Spearman $r = 0.2049$ and P value (two-tailed) = 0.4148).

The result also showed a significant difference in the plasma level of miR-208b between the survivor and nonsurvivor groups in AMI patients (Figure 1). The plasma level of circulating miR-208b in nonsurvivors was 2.1-fold higher than that in survivors (Figure 1).

3.3. Survival Analysis. The influence of the clinical characteristics on the median and 6-month survival is presented in Table 2. Only the relative expression of miR-208b ($P = 0.02$) was a significant prognosticator. Table 3 presents the results from the Cox regression analyses regarding the

Table 2: Clinical variables as predictors of the survival all AMI patients and differentiated into lower and upper 12.38 subgroups ($N = 21$, 17, and 4, resp., univariate analyses; log-rank test) in 21 AMI patients.

Characteristics	Patient N (NOE)	Median survival (days)	6-month survival (%)	P value
Relative expression				
<12.38	17 (3)	NR	66.2	**0.02**
≥12.38	4 (4)	43	0	
Gender				
Female	6 (1)	182	75	0.32
Male	15 (6)	NR	36.7	
Smoking				
Yes	8 (3)	182	47	0.81
No	13 (4)	157	37.5	
Diabetes				
Yes	4 (1)	NR	75	0.69
No	17 (6)	182	34.3	
Hypertension				
Yes	13 (5)	182	42.7	0.75
No	8 (2)	157	43.8	
Hyperlipidemia				
Yes	10 (4)	NR	52.5	0.32
No	11 (3)	182	45.5	
Decrease in EF *				
Yes	6 (2)	182	41.7	0.85
No	13 (3)	NR	51.3	

NOE: number of events; NR: not reached. *Cutoff = 0.55.

Table 3: Results of Cox regression analyses for clinical variables and miR-208 relative expression among AMI patients.

Characteristics	HR	95% CI	P value
Age	**1.04**	**(0.98–1.10)**	**0.15**
Cardiac troponin T (ng/mL)	**0.97**	**(0.87–1.05)**	**0.33**
Relative expression			
<12.38	Reference		**0.03**
≥12.38	5.08	(1.13–22.82)	
Gender			
Female	Reference		0.35
Male	2.81	(0.33–23.91)	
Smoking			
No	Reference		0.81
Yes	1.21	(0.27–5.48)	
Diabetes			
No	Reference		0.69
Yes	0.66	(0.08–5.48)	
Hypertension			
No	Reference		0.75
Yes	1.30	(0.25–6.77)	
Hyperlipidemia			
No	Reference		0.33
Yes	2.16	(0.46–10.13)	
Decrease in EF *			
No	Reference		0.85
Yes	1.19	(0.19–7.53)	

HR: hazard ratio. *Cutoff = 0.55.

clinical variables and their impact on the survival. All the variables showed no significant relation with survival in univariate analyses except the relative expression of miR-208b. To compare survival function according to levels of miR-208b, we put a cutoff point of 12.38 which was obtained by using the Youden index. High relative expression of miR-208b was the most significant negative prognostic factor in our patient cohort (HR: 5.08; 95% CI: 1.13–22.82; $P = 0.03$) (Figure 2).

4. Discussion

In this study, we investigated the prognostic value of miR-208b to predict the 6-month survival time for patients with ST-elevation myocardial infarction (STEMI). qRT-PCR analysis confirmed that baseline plasma levels of miR-208b were greater in AMI patients compared with healthy controls. An important finding in this study was that the levels of miR-208b on admission had a significant ability to predict 6-month survival time. The survival curves indicated that a relative expression cutoff of 12.38 for circulating miR-208b clearly distinguished the survival odds.

The diagnosis and treatment of AMI patients is of paramount importance, and predictive factors could have widespread application in clinical practice [2]. Cardiac troponins and creatine kinase-MB are the most common biomarkers for AMI diagnosis. However, their detection may be limited in some cases. Thus, measuring the levels of circulating miRNAs might provide an additional specific biomarker for the diagnosis and treatment of AMI. In addition, higher plasma levels of miR-208b have also been significantly associated with the risk of death during a 6-month follow-up in acute coronary syndrome (ACS) [30]. Thus, a combination of plasma miR-208b detection with clinical characterization may give greater prognosis of 6-month survival in patients with different types of heart disease.

miR-208b is encoded by intron 31 within the MYH7 gene and regulates the expression of its host gene via the Sox6 transcription factor. The MYH7 gene encodes the beta- (β-) myosin heavy chain 7 that is found in the heart (cardiac) muscle [31]. MiR-208b is considered a cardiac-specific miRNA with an important role in human heart function and cardiopathology [32]. During the early stages of AMI, this miRNA might leak out of the necrotic myocardium and be released into the circulation [33]. Cardiomyocyte-enriched miRNAs have been recently considered as the potential diagnostic biomarkers in AMI due to their rapid release, cardiac selectivity, and plasma stability [30]. Previous

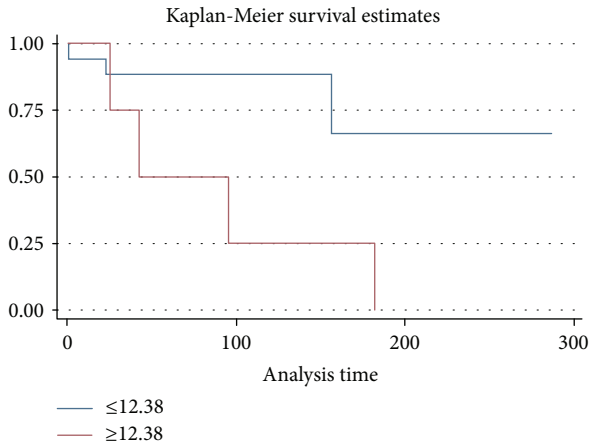

Figure 2: Kaplan-Meier curve displaying the survival in relation to high or low miR-208b relative expression.

studies reported that the plasma level of some miRNAs such as miR-1, miR-133a, miR-133b, miR-208a, and miR-499 was significantly elevated in patients with STEMI compared to healthy controls and patients with chest pain but normal coronary angiograms [32–34].

Plasma levels of miR-208b have been previously studied in relation to their predictive value in AMI [35]. Plasma miR-208b levels were higher in AMI patients, and ROC analysis gave AUC values of 0.72. An even greater predictive ROC value (0.94) for miR-208b in AMI was reported by Corsten and colleagues [33]. Several other studies have reported elevated miR-208b levels in AMI compared with healthy control subjects which have implied a role for miR-208b as a diagnostic marker [36–38]. However, none of these studies considered the rate of survival post-AMI.

A rapid increase in the level of circulating miR-208b after myocardial infarction is correlated with decreased systolic function, increased rejection fraction, and increased expression of markers of cardiomyocyte necrosis [39]. It is plausible that following myocardial damage, cardio-enriched miRNAs such as miR-208b are released into the bloodstream from necrotic cardiomyocytes which subsequently have a paracrine effect on the heart [40]. For example, miR-208b may exacerbate the deleterious conditions within the myocardium post-MI and increase the risk of death or development of heart failure [41]. As such, strategies designed to inhibit miR-208b may be of therapeutic value. In support of this concept, inhibition of miR-208a improves cardiac function and survival during heart failure [31] in addition to acting as a potential noninvasive biomarker of myocardial injury [31].

The effect of age and age-related diseases such as cancer and cardiovascular disease on the expression of circulating miRNAs has been previously examined [42]. An age-dependent decrease in miRNA expression in peripheral blood mononuclear cells (PBMCs) was seen along with lower serum levels of miR-151a-5p, miR-181a-5p, and miR-1248 [42–44]. Our data failed to show a clear effect of age on miR-208b expression, but further studies using subjects with a greater age range should be investigated.

5. Conclusion

Our result confirms previous studies demonstrating a possible role of miR-208b as a candidate biomarker for AMI diagnosis [27] and extends this to show that the precise level of miR-208b in these patients on admission was a good indicator of 6-month survival. Thus, the relative expression of miR-208b was significantly increased in AMI patients who died within 6 months compared to those AMI patients who survived, but larger studies are required to confirm this.

5.1. Limitations. There were some limitations to our study. Our findings are based on a small sample size, and further research with a larger sample size with a longer follow-up time is required to obtain accurate and reproducible results. Repeated measurements of miR-208b levels at more time points would also enhance the reliability of our findings.

Data Availability

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

Conflicts of Interest

There is no conflict of interest among the authors.

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