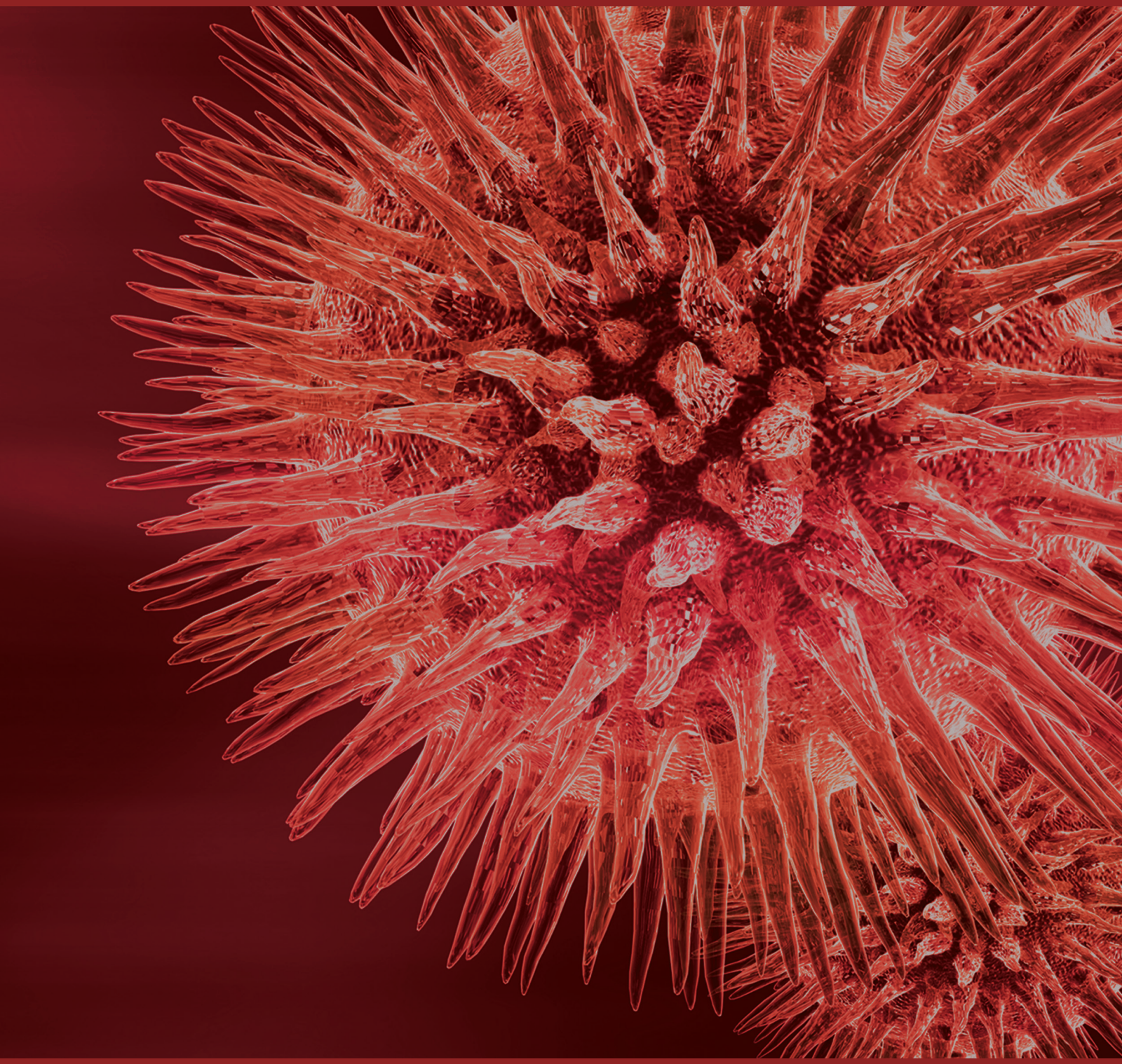
BioMed Research International

Cardiac Proteomics

Guest Editors: Jatin G. Burniston, Anthony O. Gramolini, and R. John Solaro



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Contents

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Editorial

Cardiac Proteomics

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Approximately one-third of all deaths are attributed to cardiovascular diseases, and ischaemic heart disease is the primary cause of death globally. Hence there continues to be a pressing need for advances in preventative, diagnostic, and interventional strategies for cardiovascular disease. The multifactorial nature of cardiovascular disease, which encompasses both genetic and environmental factors, presents a major challenge to research in these areas. Furthermore, personalised strategies, which are anticipated to hold the key to reducing cardiovascular mortality rates, require yet more sophisticated biomarkers capable of accurately predicting disease risk, informing clinical decision-making, or monitoring therapeutic responsiveness.

Omic approaches, including genomics, transcriptomics, proteomics, and metabolomics, exploit genome information and use high-throughput analysis techniques. Because the data are comprehensive and the measurements are performed in parallel (i.e., reducing interassay variability), it lends itself to interrogation by bioinformatic tools that provide objective "unbiased"/"unsupervised" assessment of pertinent features, functional clusters, interactions, and regulatory networks. The marriage between comprehensive data collection and computational analysis is proven as powerful and pragmatic means of discovering new information and candidate biomarkers, which might not otherwise have been investigated. This editorial aims to orientate the reader with regard to where proteomic studies sit amongst the other levels of investigation (i.e., genomics, transcriptomics, etc.) and draw attention to the particular strengths and challenges involved in proteomic work.

The heritability of cardiovascular disease is estimated to be approximately 40%, but large multinational efforts to link disease risk to specific genetic variations have not yet identified high-penetrance genes with large effects. Indeed, the top 34 candidate genes explain less than 15% of the variance in heritable disease risk, and each gene has small (<5%) individual effects (reviewed in [1]). This emphasises the complex polygenetic nature of cardiovascular disease and also highlights the prominent influence that environmental factors have in determining cardiovascular risk. Because the genome is essentially static, genomic studies are not best placed to capture interactions with the environment. On the other hand, the transcriptome is cell-specific and can be influenced by epigenetic modifications and environmental inputs. Moreover, the technology for monitoring gene expression is particularly advanced and high-throughput arrays can be performed on a genome-wide scale, which provides an unsurpassed breadth of information.

Notwithstanding the strengths of transcriptome tools, gene expression (i.e., mRNA abundance) cannot be assumed to be associated with similar changes in the abundance or activity of their products (i.e., proteins). In part, this is because the net abundance of a protein is the result of the balance between its rates of synthesis and degradation and cannot be predicted solely from its mRNA transcript. In addition, posttranscriptional processing, microRNA interference and ribosomal capacity each affect the translation from mRNA to protein. Most important of all the activity of proteins, their cellular localisation and interaction with other proteins are regulated by a multitude of posttranslational modifications,

including phosphorylation, acetylation, and glycosylation, which may combine to create numerous different "protein species" or "proteoforms" from each gene. Because of this, the number of proteins far exceeds the number of expressed genes and there is a wealth of information that can only be obtained by studying the proteome.

The protein complement of the heart directly underpins its functional properties, including electrophysiology, contractility, susceptibility to disease, and response to pathological insults. Moreover, the proteome is the interface between the environment and the genome. Signals from the environment are transduced through the proteome and may affect transcription factors, ribosomal capacity, or the activity of degradative processes to bring about changes (in the proteome) that alter the functional properties of the cell. The intimate link between cardiac physiology and the protein complement of the heart makes the proteome a highly fertile ground for discovering biomarkers to predict disease susceptibility, assist diagnoses, or help to monitor responses to therapeutic interventions. Indeed, the great strength of established prediction tools such as Framingham Risk Score lies in their ability to capture the interaction between an individual's genome and their environment, including physical activity level, diet, and other stressors. Therefore, proteome studies are well placed to provide the next generation of clinical biomarkers.

While the potential fruits of proteomics are great, so are the challenges. Not only is the proteome more expansive than any other biological level, but also proteins exhibit a broad range of different physicochemical properties, which affect their solubility and make it difficult to extract all proteins using a single technique. Moreover, the dynamic range of protein abundance spans at least 7 orders of magnitude [2], and the proteins cannot be amplified and the entire proteome is not known. In particular, proteoforms cannot be predicted and must be identified and characterised empirically, and cataloguing of proteins using mining techniques (e.g., [3, 4]) is an important aspect of cardiac proteomics work. Unlike nucleic acids, interaction between proteins is not based on well-understood complimentary binding so it is difficult to predict protein-protein interactions, binding partners, substrates, and so forth. To address these and other challenges, different proteomic techniques have been developed for descriptive and comparative studies. Nonetheless, proteomic studies share common building blocks including proteome separation, mass spectrometry analysis, and protein identification (Figure 1).

Conceptually, proteomic investigations are described as either "bottom-up" or "top-down." Top-down studies such as those of S. Yavuz et al. and D. Zheng et al. typically employ 2-dimensional gel electrophoresis (2DGE) to spatially separate proteins according to their isoelectric point (pI; first dimension, isoelectric focusing) and relative molecular mass (M_r; second dimension, denaturing gel electrophoresis). Image analysis is used to discover differences in spot volumes between case and control samples. Then, gel spots are excised and the proteins digested with trypsin and analysed by mass spectrometry to identify the proteins of interest. Hence, the workflow begins at the protein level and works down to the peptide level, that is, top-down. This sequence also illustrates the open/discovery philosophy of proteomics, wherein differences between samples are detected before the protein is identified, which is diametrically opposite to hypothesis-led reductionist designs where the target of interest must be defined *a priori*.

S. Yavuz et al. report 2DGE analysis of pericardial fluid and highlight omentin-1 as a candidate biomarker that may assist in the classification of pericarditis. Currently, more than one-quarter of pericarditis cases are designated as idiopathic based on lack of classification by current biochemical techniques. Mining of pericardial fluid proteome has been described [5] but few other studies report comparative analysis of this important body fluid. D. Zheng et al. also address the need for biomarkers directly in human clinical samples using a top-down approach. Rheumatic heart disease, which is a serious problem in developing countries, is difficult to diagnose because patients often do not present typical cardiovascular risk factors. D. Zheng et al. used 2DGE to show that cardiac samples of rheumatic heart disease have a drastically greater abundance of heat shock protein 60 compared to matched samples from patients with mitral valve prolapse. What is evident from each of these studies is that the novel biomarkers fit well with our current mechanistic understanding of these diseases.

While 2DGE offers robust comparative analysis of protein species, standard gel systems have limited ability to resolve proteins larger than ~150 kDa or proteins at the extremes of the pI scale (i.e., <pH 4 or >pH 9). Bottom-up workflows overcome these issues by digesting the sample into peptides, which have fewer solubility issues. Typically, peptides are resolved using reverse phase liquid chromatography (RPLC) and tandem mass spectrometry, which records peptide ion masses and their fragment-ion spectra. Using this approach, differences between case and control are determined based on mass spectrometry data of either the relative intensity (ion abundance) or number (spectral counting) of the peptide ions. Alternatively, samples can be labelled using differential mass-tags, which enables groups of samples to be combined and analysed equivalently. The major challenge in this workflow lies in the data analysis. In particular, peptides must be unambiguously linked to their parent protein and, ideally, isoform-specific peptides should be used for quantitation; otherwise, differences must be described for the group of proteins to which those peptides could belong.

S. Abdul-Ghani et al. report cardiac phosphoproteome responses to remote ischaemic preconditioning (RIPC) in mice using a peptide labeling bottom-up technique. The protective effects of RIPC have been convincingly demonstrated but the underlying mechanisms have yet to be identified (reviewed in [6]). Phosphopeptide analysis is particularly challenging because the number of phosphorylated copies of a protein is usually a relatively small proportion of the total. Therefore, selective enrichment strategies such as TiO_2 affinity are used. This strategy enabled S. Abdul-Ghani et al.

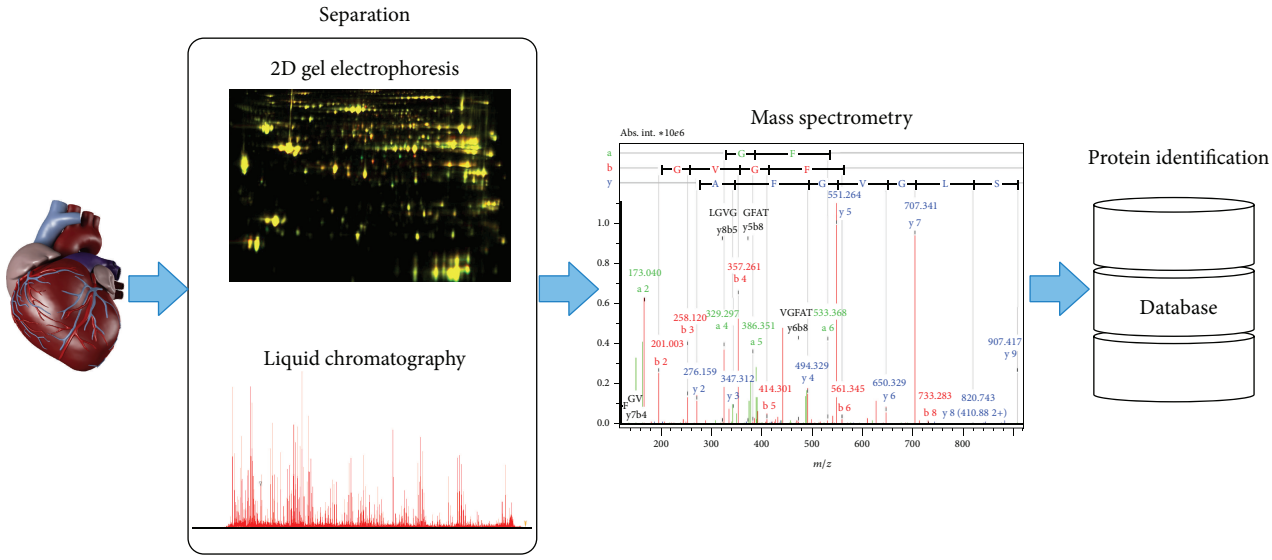

FIGURE 1: Proteomic workflow. Proteomic experiments share a common workflow encompassing separation, mass analysis, and protein identification. The top-down approach uses image analysis of proteins separated by 2-dimensional gel electrophoresis to find differences between case and control samples. Protein spots of interest are then identified by comparing their peptide mass spectrometry data to protein databases. Conversely, bottom-up workflows first digest proteins extracted from cardiac samples into peptides. The peptides are separated by reverse-phase liquid chromatography, and mass spectrometry is used to identify and quantify the proteins. Common to each of these strategies is the philosophy that differential analysis is performed prior to protein identification. This purposeful "open" approach enables proteomic work to discover information that might not otherwise have been hypothesized.

to discover a novel site-specific phosphorylation of the Z-disc protein, myozenin-2, which may have a signalling role in RIPC cardioprotection. L. E. de Castro Brás et al. also report the use of a bottom-up technique and investigated age-associated differences in the extracellular matrix composition of mice lacking the SPARC gene. SPARC (secreted protein acidic and rich in cysteine) is a matricellular protein involved in the assembly of collagen fibrils. Loss or gain of the function of SPARC impacts cardiac fibrosis, and L. E. de Castro Brás et al. used orthogonal separation processes (SDS-PAGE and RPLC) and spectral counting to reveal that SPARC may contribute to age-associated cardiac stiffening.

In addition to the aforementioned original research articles, this special issue includes comprehensive reviews of hypertension- and exercise-related cardiac adaptations, presented by B. A. Petriz and O. L. Franco, and dystrophinopathy-associated cardiomyopathy, presented by A. Holland and K. Ohlendieck. These reviews bracket the continuum spanning from monogenic disease to complex polygenic traits and highlight how proteomic strategies are being used to advance these fields. We hope this special issue provides readers with new insight to cardiac research and the role that proteomics can play in generating novel information and candidate biomarkers.

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Review Article

Effects of Hypertension and Exercise on Cardiac Proteome Remodelling

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Left ventricle hypertrophy is a common outcome of pressure overload stimulus closely associated with hypertension. This process is triggered by adverse molecular signalling, gene expression, and proteome alteration. Proteomic research has revealed that several molecular targets are associated with pathologic cardiac hypertrophy, including angiotensin II, endothelin-1 and isoproterenol. Several metabolic, contractile, and stress-related proteins are shown to be altered in cardiac hypertrophy derived by hypertension. On the other hand, exercise is a nonpharmacologic agent used for hypertension treatment, where cardiac hypertrophy induced by exercise training is characterized by improvement in cardiac function and resistance against ischemic insult. Despite the scarcity of proteomic research performed with exercise, healthy and pathologic heart proteomes are shown to be modulated in a completely different way. Hence, the altered proteome induced by exercise is mostly associated with cardioprotective aspects such as contractile and metabolic improvement and physiologic cardiac hypertrophy. The present review, therefore, describes relevant studies involving the molecular characteristics and alterations from hypertensive-induced and exercise-induced hypertrophy, as well as the main proteomic research performed in this field. Furthermore, proteomic research into the effect of hypertension on other target-demerged organs is examined.

1. Introduction

Hypertension is the main risk factor for cardiovascular diseases, which include stroke, coronary artery disease (CAD), and heart failure (HF) leading to ~1.8 million deaths worldwide every year [1]. Moreover, essential hypertension results from the interaction of pathological mechanisms, environmental factors, and a complex genome background [2]. Cardiac pathological hypertrophy is one of the main phenotype adaptations to hypertension. Complex molecular signalling marks this process, which is transcripted to an altered cardiac proteome. Pressure overload cardiac hypertrophy is thus often marked by dysfunction within cardiac function, which, over time, may turn into HF [3, 4].

The pathogenesis of hypertension and its pathophysiology have been widely investigated by several genomic approaches, which include analysis of candidate genes and high-throughput genetic mapping such as complex genome-wide scans [5, 6]. These strategies have also been integrated with functional physiological genomics to better understand the physiological responses resulting from gene expression and their biological interactions [7, 8]. To date, proteomic strategies have been used as a complementary tool into the investigation of the pathophysiological effects of hypertension rather than its pathogenesis.

Left ventricle hypertrophy is one of the main outcomes of pressure overload stimulus [9, 10]. This phenotype modification is driven by a complex modulation within the cardiac proteome that is still being widely investigated, since the molecular mechanism underlying this process is still not fully elucidated. Despite some morphological similarities, pathological and physiological cardiac hypertrophies are characterized by a distinct genome and proteome profile [11–13]. Moreover, it has been suggested that exercise stimulus may reduce the onset of pathological cardiac hypertrophy in hypertension, being also indicated to attenuate

cardiac maladaptation thought the systematic reduction in blood pressure [14–18]. However, the effect of exercise on the hypertensive myocardium lacks more experimental and comparative proteomic data. This review therefore provides an overview of proteomic research into cardiac proteome remodelling in hypertension and exercise stimulus.

2. An Overview of Hypertension and Cardiovascular Diseases

Hypertension is a multifactor disease characterized by chronic elevation in blood pressure to levels equal to or above 140 mmHg systolic blood pressure (SBP) and above 90 mmHg of diastolic blood pressure (DBP) [1]. Considered a worldwide epidemic disease, hypertension is the main risk factor for cardiovascular disease [19], being epidemiologically closely associated with metabolic diseases such as obesity and diabetes [20]. Cardiovascular disease leads to ~17 millions of death per year, and, from this total, it is reported that high blood pressure is estimated to cause more than half of these deaths (over 9 million deaths every year), making it also the main risk factor in the global disease burden [21]. Well-known causes of the pathogenesis of hypertension account for approximately 5% of the cases; these involve alteration in renal salt-water homeostasis, hyperstimulation of the sympathetic nervous system, hormone dysfunction, and single gene mutation [2, 22]. Thus, the development of hypertension is attributed to multifactorial and unknown factors [2]. Indeed, the pathogenesis of essential hypertension is most likely to result from the association of several pathophysiological stimuli (e.g., obesity and diabetes) with environmental factors (e.g., diet, lifestyle, tobacco, and alcohol abuse) and genetic background [23], with hereditability estimated at 15–40% [22].

Hypertension and other pathologies, such as obesity and diabetes, together with environmental factors such as physical inactivity, diet (e.g., hypercaloric and alcohol abuse), and tobacco are likely to enhance cardiac insults [24]. These risk factors may lead to vascular dysfunctions (e.g., dysfunction in endothelial vasodilation and artery stiffness) which, if not treated, progress to cardiac damage [25]. Moreover, systemic high blood pressure leads to several impairments in cardiac apparatus, especially in relation to cardiac hypertrophy (described in the following topic). The chronic overload on the myocardium is associated with the development of heart dilation and contraction impairment [11]. When it results from hypertension or cardiac congenital pathology, cardiac hypertrophy may progress into heart failure and it can be an independent risk factor for other cardiac conditions such as myocardial infarction and arrhythmia [4, 26, 27].

Hence, the identification of the molecular mechanisms involved in cardiac hypertrophy in response to pressure overload is of prime importance to understand the pathophysiology of hypertension for the myocardium and for transition to heart failure. Moreover, the investigation of the distinct molecular regulation of pathological and physiological hypertrophy (e.g., in response to exercise stimuli) may also contribute to identifying new therapeutic targets and to a better understanding of how exercise may prevent and attenuate pathological stimuli such as hypertension.

3. A Brief View of Cardiac Remodelling: Pathological versus Physiological Stimuli

Cardiac enlargement occurs mainly due to an increase in myocyte size, which is triggered by several events, including increased functional load on myocyte, activation of signalling pathways and gene expression, upregulation of protein synthesis, and formation of novel sarcomeric units [11]. Moreover, this process seems to be triggered by a mechanosensing mechanism in cardiac myocytes through stretch-sensitive ion channels, growth factor receptors, and *G*-protein-coupled receptors, linking stress and pressure overload stimulus to gene regulation and protein synthesis [3, 11].

These molecular mechanisms are responsible for cardiac growth, a natural physiologic process, seen in the postnatal period until the heart reaches its natural size in adulthood [3]. Cardiac remodelling may also occur in response to external stimulus, which promotes heart hypertrophy such as pregnancy [28, 29] and exercise [30, 31] or as an outcome of pressure overload (e.g., aortic stenosis and systemic blood pressure) and cardiomyopathies (e.g., mutations in sarcomeric genes and associated diseases) leading to pathological cardiac hypertrophy [11]. Moreover, physiologic and pathologic cardiac hypertrophy display a distinct molecular signature resulting in a distinct cardiac phenotype [11, 32]. While physiologic hypertrophy is associated with improved cardiac function, pathologic hypertrophy is often associated with myocyte loss, fibrosis, alteration in myocyte metabolism (shift from fatty acid oxidation to glucose metabolism), and cardiac dysfunction [3, 11]. Moreover, in contrast to physiologic hypertrophy, pathological cardiac remodelling is characterized by an irreversible phenotype status [11, 33]. For a detailed view of the molecular mechanisms underlying cardiac hypertrophy see [3, 11, 34, 35]. Figure 1 presents the main alterations in heart and myocyte morphology due to pathological and physiological stimuli.

4. Hypertension and Pressure Overload Factors for the Cardiac Proteome

Rapid advances in the genomic field have led to a large amount of data in hypertension research, ranging from the analysis of several candidate genes to high-throughput genetic mapping (e.g., complex genome-wide scans) [5, 6]. It has been stated that the genomic approach is likely to investigate the pathogenesis of hypertension rather than its pathophysiology [36]. Moreover, functional genomic analysis and, more recently, proteomics, have been widely used to better understand the pathophysiology of hypertension. In this regard, the great advance of proteomics, as a postgenomic tool, is its ability to identify gene products, including post-translational modifications (PTM), and further investigate the expression of these protein species for phenotype and physiological responses [37].

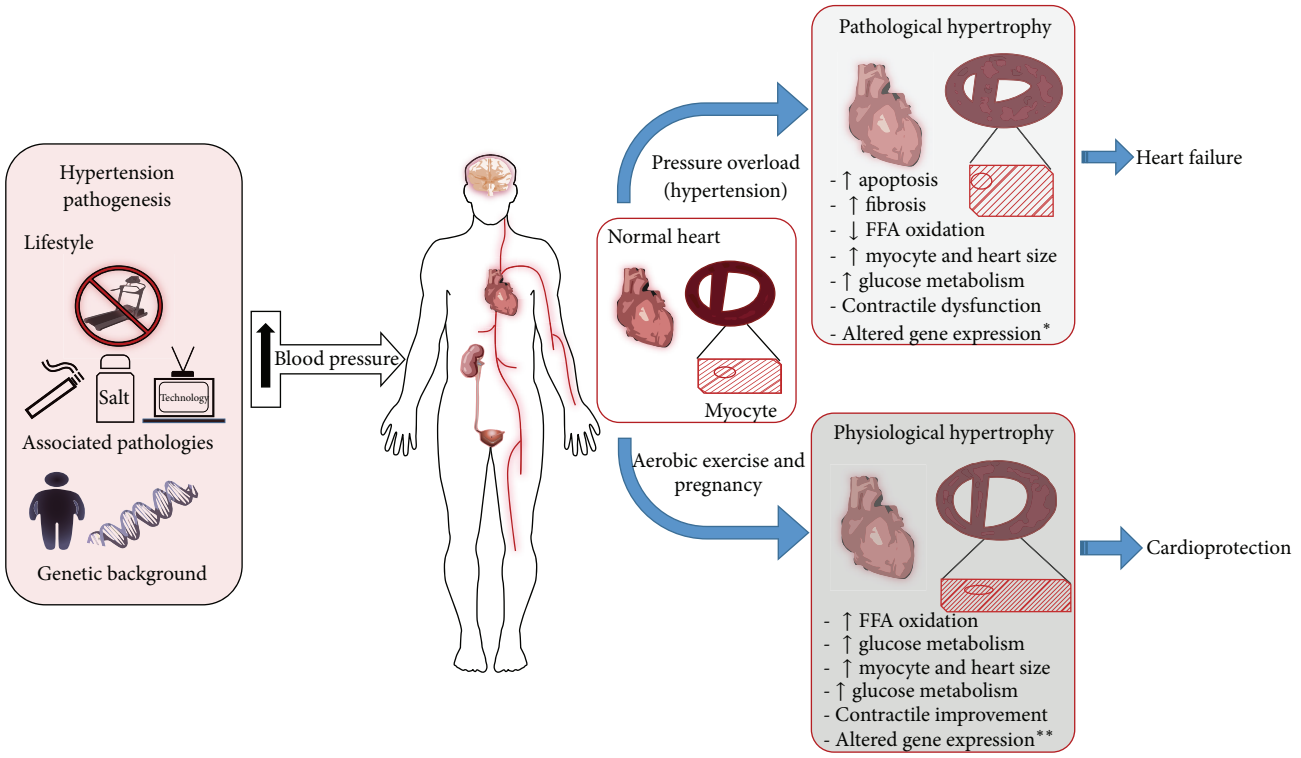

Figure 1: Pathologic and physiologic cardiac hypertrophy. Figure 1 sums the factors associated with hypertension pathogenesis and its effect on some target organs (e.g., brain, kidney, and arterioles: highlighted) and cardiovascular system. Moreover, differences in cardiac hypertrophy, heart transversal session, and cardiomyocyte are presented between pathologic and physiologic hypertrophy, followed by distinct physiologic and molecular regulations. Distinct molecular regulation between pathologic and physiologic cardiac hypertrophy is associated with the development of cardiac dysfunction* or cardiac improvement**.

Pressure and stress overload lead to transcriptome regulation and triggering changes in the cardiac proteome [38]. Differences in the cardiac 2-DE proteome pattern between nonhypertensive (Wistar-Kyoto rats) and spontaneously hypertensive rats (SHR) [39] support this, while several other studies show modulations in the heart proteome followed by pressure overload hypertrophy [40]. Although heart adaptation to pressure overload is widely adverse, this molecular signalling dictates asymptomatic phenotype modulations, which over time affect cardiac structure (e.g., LV hypertrophy) and function (e.g., contractile impairment) and often evolve into heart failure [4] Figure 1.

In this regard, several experimental models have been used to better understand the effect of hypertension and other pressure overload effects on the cardiovascular system and heart tissue. Moreover, spontaneous development (SHR), transgenic (dTGR: double transgenic rats harbouring human renin and angiotensin genes) and mechanical induction (e.g., aortic constriction) of hypertension are widely used for this purpose [41–43]. Hypertrophic-inducing agents (e.g., ET-1, Ang II, and isoproterenol) are also often used in cardiomyocytes to investigate the molecular mechanism and signalling pathways underlying physiologic and different types [39] of pathological hypertrophy [11]. However, it is observed that several of these studies combine physiological observations with biochemical and genomic data, lacking proteomic information. Proteomic research could therefore provide more insights into the molecular events within the cardiac hypertrophy phenotype. Accordingly, this section will describe some relevant proteomic studies from this perspective. Moreover, proteomic workflow and protein targets associated with cardiac hypertrophy are shown in Figure 2.

As mentioned before, left ventricle hypertrophy (LVH) is a well-known characteristic of cardiac adaptation to pressure overload and an essential criterion of hypertensive heart disease [44]. Studies have shown that the LV proteome in particular is highly altered in this process, even at the early stages of hypertension [45]. The spontaneously hypertensive rat (SHR) is one of the main experimental models of essential hypertension, displaying several characteristics of this pathology, including LVH [46]. In this experimental model, research has highlighted the role of protein phosphorylation as a molecular signature common to the pathogenesis of cardiac hypertrophy [40, 47].

Furthermore, phosphoproteins such as α-enolase, SR-Ca^{2+}-ATPase, and phospholamban have been shown to be crucially associated with cardiac hypertrophy induced by hypertension in SHR [40, 47]. In this regard, LV proteins from SHR and control (Wistar-Kyoto) rats were enriched for phosphoproteins (phosphoaffinity chomatography column) and then analysed by 2-DE, followed by phosphoprotein specific staining (Pro-Q diamond) identification by MALDI

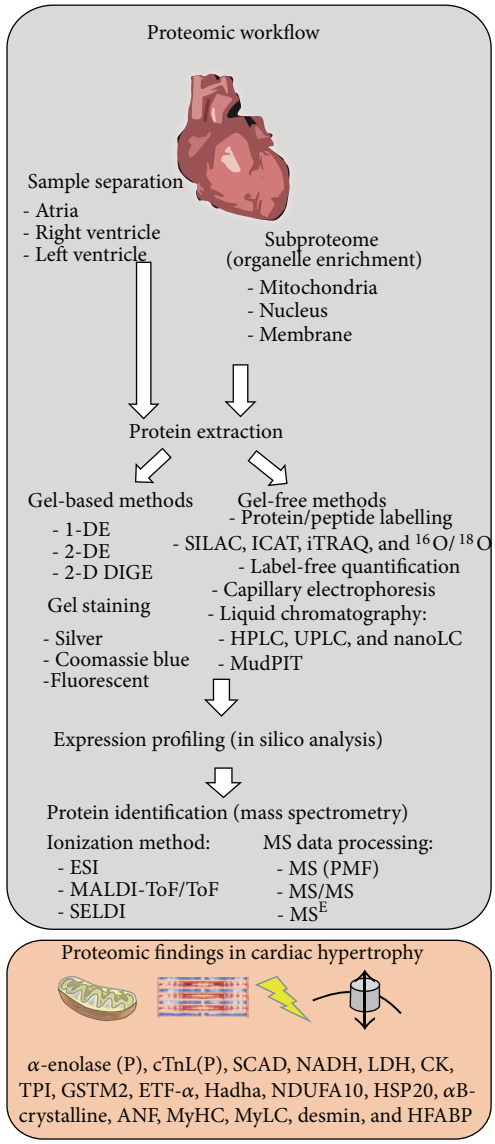

Figure 2: Workflow in cardiac proteome research. Figure 2 presents an overview of proteomic tools that may be used in cardiac proteome research. Starting by samples separation where heart tissue may be separated according to the research interest, followed by total protein extraction or subproteome profiling (e.g., organelle enrichment). Moreover, after protein extraction, several proteomic tools (e.g., gel-based and gel-free) may be used for qualitative and/or quantitative (relative and/or absolute) proteome analysis and identification through mass spectrometry (MS). Lower panel indicates some protein targets (metabolic, contractile, stress-, and signalling related) associated with cardiac hypertrophy or modulated by hypertrophic process. α-enolase(P) (phosphorylated alpha-enolase) and cTnL(P) (phosphorylated cardiac troponin I), SCAD (short-chain acyl-CoA dehydrogenase), NADH (nicotinamide adenine dinucleotide), LDH (lactate dehydrogenase), CK (creatine kinase), TPI (triose phosphate isomerase), GSTM2 (glutathione S-transferase Mu 2), ETF-α (electron transfer flavoprotein-alpha), Hadha (3-hydroxyacyl-coenzyme A dehydrogenase), NDUFA10 (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10), HSP20 (heat shock protein 20), αB-crystalline, ANF (atrial natriuretic peptide), MyHC (myosin heavy chain), MyLC (myosin light chain), desmin, and HFABP (heart-type fatty acid binding protein).

TOF [40]. Here, 21 protein spots were significantly altered between groups where 19 proteins were identified as being related to metabolism, contraction, cell cycle, and signalling. Multiple phosphorylations were also observed, with attention to 3-ketoacyl-CoA thiolase, which had not been previously shown to be phosphorylated. In this study, close attention was paid to the hyperphosphorylation of α-enolase in SHR, which was also seen in younger SHR (4 weeks old, data not shown) but was not present in the right ventricle or in atria. Authors have shown that four-week-old SHR did not develop hypertension, indicating that the hyperphosphorylation of α-enolase may not be secondary to hypertension. Moreover, in the present study it was shown that α-enolase enzymatic activity is reduced by phosphorylation in LV. These data seem to be inconsistent with the literature, where anaerobic glycolysis is shown to be enhanced in several models of

cardiac hypertrophy [11, 48]. Thus, it is speculated that hyperphosphorylation of α-enolase in LV of SHR may display another function beyond its catalytic activity.

The LVH proteome has also been investigated in two different animal models of hypertension to verify key proteins related to hypertensive hypertrophy [49]. In this study, the LV proteome from SHR (model of essential hypertension), renovascular hypertensive rats (RHR, a model of secondary hypertension made by clipping renal arteries), and control rats (Wistar-Kyoto) was shown to present a distinct proteome profile. Two-D DIGE MALDI TOF detected 29 protein spots with a significant difference in expression (2-fold) among the groups (20 spots between RHR and SHR, 23 between SHR and control rats, and 19 between RHR and control rats). From this total, 18 protein spots were identified belonging to 16 unique proteins (including different isoforms and posttranslational modifications). Moreover, glutathione-S-transferase (GSTM2) and short-chain acyl-CoA dehydrogenase were (SCAD) both downregulated in SHR but not in RHR, compared with control animals; results were confirmed by Western blot, RT-PCR, and enzymatic activity. A different pattern was seen in LVH in both models, which may result from the distinct proteome profile seen in this study, where GSTM2 and SCAD may be relevant candidates in the development of LVH in SHR. Moreover, also it was shown that LVH regressed by pharmacologic means still maintains the proteomic characteristics of hypertrophied hearts [50]. This was shown by 2-DE MALDI-TOF analysis, where 53 protein spots (related to 36 unique proteins) were altered in hypertensive hearts (e.g., upregulation of SCAD, NADH, enolase 1α, and aldehyde dehydrogenase and downregulation of ETF-α, superoxide dismutase, and thiol-specific antioxidant). The authors showed that antihypertensive treatment led to normalization of proteins related mainly to contractive and stress-related processes, but those 17 proteins with an essential role in energy production, cell stress defence, and hypertrophy regulations remained unchanged after LVH regression.

The role of myocardial K_{ATP} channels in cardiac hypertrophy has been widely investigated to date [51–53]. K_{ATP} channels are ATP-sensitive channels formed by four pore Kik6.2 subunits and four regulatory SUR1 subunits, known to present cardioprotective properties, due to their integration with other myocyte protein channels and proteins associated with cellular bioenergetics pathways, playing a prominent role in metabolic homeostasis [52]. Research has shown that deficiency in myocardial K_{ATP} channels is currently thought to play a role in hypertension pathophysiology [54, 55]. Comparative 2-DE analysis followed nanoelectrospray LC-MS/MS [53], and Orbitrap MS protein identification [51] found that an experimental model lacking Kir6.2 ATP-sensitive K(+) (K(ATP)) channels generates unfavourable cardiac proteome remodelling in hypertensive myocardium. Both studies have shown that over 170 proteins presented a significant differential expression in response to dysfunction of K_{ATP} channels, with 95 proteins being linked with metabolic function (e.g., lactate dehydrogenase, SCAD, pyruvate kinase, triosephosphate isomerase, and creatine kinase), and they are also associated with bioenergetic enzymes that were previously linked to K_{ATP} channel activity in other studies [52]. Thus, because Kit6.2, an isoform of cardiac K_{ATP} channels, is associated with stress adaptation within the myocardium, dysfunction of K_{ATP} channels is thought to underlie heart disease [52, 56].

Proteinases seem also to be a relevant class of proteins in the pathophysiology of hypertension, due to their central role in blood pressure control among other vital physiologic functions such as coagulation [57, 58]. In this way, MS-based proteomics is a robust tool in the research of the complex protease network such as the renin-angiotensin system (RAS), a widely investigated proteolytic network with a central role in hypertension development [59–61]. Moreover, the RAS also acts in a tissue-specific way (e.g., brain, skeletal muscle, kidney, and myocardium), presenting distinct local physiological responses [62]. The heart's local RAS is known to be stimulated by hemodynamic stress (e.g., pressure and volume overload), where angiotensin II is the main vasoactive product of this system, and also known to modulate contractile-related molecular expression (skeletal α-actin, β-myosin heavy chain, atrial natriuretic polypeptide, and fibronectin) and promote cardiac phenotype remodelling [63] and hypertrophy [41, 64]. Otherwise, inhibition of Ang II by angiotensin converting enzyme inhibitors (ACEI) attenuates cardiac hypertrophy induced by pressure overload in experimental models and humans [62, 65], and it has been established that the inhibition of RAS attenuates and regresses cardiac hypertrophy induced by hypertension [66]. Moreover, Ang II receptors, AT_1 and AT_2, have been widely investigated as intermediates for pathological stimuli in the cardiovascular system, where the stimulation of AT_1 (a *G*-protein-coupling receptor) is shown to trigger vasoconstriction signalling [62, 67] and cardiac hypertrophy through the activation of mitogen-activated protein kinase (MAPK) and protein kinase (PK) [68].

Heart mitochondrial proteome profiling by LC-MS/MS analysis in dTGR (double transgenic rats harbouring human renin and angiotensin genes) after caloric restriction (60% of energy intake for 4 weeks) revealed seven differential proteins compared to dTGT without caloric restriction. Moreover, the present study identified 6 proteins (downregulation of cytoskeletal and enzyme modulators and upregulation of oxidoreductase) present only in dTGR rats compared to the other experimental groups, including Sprague-Dawley rats control group [69]. The present study also indicated that CR attenuated cardiac hypertrophy, fibrosis, and cardiomyocyte apoptosis, suggesting that modulation in the mitochondrial proteome by caloric restriction may attenuate cardiovascular disorders induced by Ang II. Besides proteomic analysis, cardiac hypertrophy induced by Ang II in the dTGR model was also shown by gas-chromatography TOF to modulate the cardiac metabolome in more than 100 metabolites [70]. Moreover, comparative label-free LC-MS/MS analysis revealed that pressure overload heart hypertrophy induced by aortic constriction led to downregulation in the abundance of mitochondrial fatty acid oxidation proteins and upregulation of pyruvate dehydrogenase subunits and tricarboxylic acid cycle proteins [71]. These data sustain the role of RAS components in cardiac remodelling induced by Ang II, as well

as the relation between mitochondrial dysfunction, altered cardiac metabolism [70] (e.g., downregulation of mitochondrial and lipid metabolism genes) [72], and the proteome as pivotal factors in cardiac pathological hypertrophy.

Despite the technical difficulty in separating cytosolic from mitochondrial proteins and other contaminants as well as determining the relevant cytosolic proteins which translocate to mitochondria during several physiological processes (e.g., apoptosis) [73], research into the mitochondrial proteome is an important issue in maladaptation in cardiac hypertrophy [3, 45]. Several data indicate mitochondrial dysfunction and impairment in cardiomyocyte metabolism as strong characteristics in overload cardiac hypertrophy [74–76]. Moreover, the altered cardiac mitochondrial proteome was recently shown to precede and contribute to the development of hypertension in spontaneously hypertensive rats [45]. In this study, authors showed by 2D-DIGE combined with MALDI TOF/TOF that prehypertensive (4-week-old rats) and further hypertensive stage (20-week-old rats) harbour distinct mitochondrial proteome in the left ventricle portion. It was observed that the prehypertensive stage presented a greater proteome alteration (significant alteration in 33 protein spots, 16 upregulated and 17 downregulated) compared to the 20-week-old SHR (13 protein spots significantly altered). In this study, the authors highlighted the alteration in mitochondrial trifunctional enzyme alpha subunit (Hadha) and dehydrogenase 1 alpha subcomplex 10 (NDUFA10) as possible relevant molecular agents in the development of cardiac hypertrophy in SHR, since both enzymes were differentially expressed as early as one week of age in this rat strain.

Myocyte hypertrophy is also stimulated by different signalling pathways through the stimulation of endothelin-1 (ET-1), which includes the protein kinase C, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase (MAPK), which also includes p38 mitogen-activated protein kinase and c-Jun N-terminal kinase pathway [77]. Endothelin-1 is a strong vasoconstrictor peptide hormone and stimulator of RAS, which is widely used to induce cardiac hypertrophy [78]. Recently, 2-DE followed by LC ESI-MS/MS analysis revealed that concentric cardiac hypertrophy induced by ET-1 revealed a distinct proteome compared to eccentric induced hypertrophy [43]. Authors found that twelve different proteins were differently expressed in cardiomyocytes treated with ET-1 compared to control non-treated cardiac cells, where eight proteins were upregulated and another three downregulated. From those, αB-crystalline, associated with cardioprotection and ANP, a biomarker for pathologic cardiac hypertrophy, presented the highest upregulations [43]. A more recent study found similar data, indicating that cardiomyocyte hypertrophy induced by ET-1 led to proteome modulation with the increase in expression of desmin protein species and αB-crystalline [79]. Other cardiac hypertrophic stimuli, such as isoproterenol (ISO), were also observed to promote an alteration in healthy cardiac tissue and in the cardiac proteome, shown by 2-DE MS/MS analysis [80]. Isoproterenol is a catecholamine widely applied in cardiovascular research as a model for adrenergic stimulation with a close association with pathological cardiac hypertrophy [81]. Here, seven proteins were differentially expressed in pathological hearts where myosin light chain 2 and 3, desmin, prohibitin, heart fatty acid binding protein, and ATP-synthase 5β were downregulated, while heat shock proteins 60, 70, and D1 were upregulated. Although some data have been shown to be contrary to previously reported studies (e.g., desmin upregulation shown by Agnetti et al. [79]), this may indicate a variation in cardiac response to something other than cardiac hypertrophy stimulus (e.g., ISO versus ET-1 hypertrophic stimuli).

Finally, the transition from pathological hypertrophy to HF makes the discovery of biomarkers for early disease treatment of HF an urgent necessity. Troponin I seems to present a high specificity for this purpose. Analysis of pathological and healthy human heart tissue by top-down MS-based quantitative proteomics has detected the phosphorylation of cTnI in Serine 22/23 sites at an early stage of CHF, making it a strong candidate biomarker for this pathologic state [82]. This study also presents top-down proteomics as a viable clinical tool in biomarker research. Moreover, the investigation of the molecular mechanisms involved in pathological hypertrophy is of great interest due to the high correlation with heart failure [26]. Although the entire molecular mechanisms underlying the development of pathological heart hypertrophy have not been fully elucidated, it has been noted that this process is coordinated by multifactorial events rather than by a single target or stimulus. Furthermore, pharmacologic and alternative strategies such as exercise may be addressed to prevent and treat pathological cardiac hypertrophy. The main alterations in cardiac proteome listed in this section are presented in Table 1.

5. Proteomic Research in Other Target Tissues

Rapid advances in the genomic field have led to large amount of data in hypertension research, ranging from the analysis of several candidate genes to high-throughput genetic mapping (e.g., complex genome-wide scans) [5, 6]. Moreover, it has been seen that the genomic approach is likely to investigate the pathogenesis of hypertension rather than its pathophysiology [36]. Functional genomic analysis, and more recently, proteomics, have both been widely used to better understand the pathophysiology of hypertension. In this regard, the main advance of proteomics, as a postgenomic tool, is its ability to identify gene products, PTM, and further investigate the expression of these protein species for phenotype and physiological responses [37]. Undoubtedly, proteomic analysis plays an important role in hypertension research, where the cardiac and vascular proteomes have been the main focus [25, 36, 82–86].

Several proteomic studies involving the pathophysiology of hypertension have been carried out in renal and vascular tissue. Among these, Thongboonkerd et al. [87] performed an elegant study evaluating the effect of hypoxia (a component of obstructive sleep apnoea, closely associated with hypertension) on the renal proteome in Sprague-Dawley rats. In this study, rats submitted to intermittent

Table 1: Cardiac proteome modulated by pathologic cardiac hypertrophy.

Experimental model	Experimental method	Main altered proteome	Reference
SHR and WKY	2D-DIGE	Comparison between different SHR age and animal models: (i) 33 mitochondrial proteins with altered expression between SHR groups; (ii) Hadha and NDUFA10 with differential patterns in SHR versus WKY.	[45]
SHR	Phosphoaffinity chromatography; 2-DE; Pro-Q staining; MALDI TOF	Protein phosphorylation in cardiac hypertrophy linked with hypertension: (i) 3-ketoacyl-CoA thiolase; (ii) α-enolase hyperphosphorilation (reduced enzymatic activity); (iii) SR-Ca^{2+}-ATPase and phospholamban.	[40, 47]
Human heart tissue	Top-down MS-based quantitative proteomics	Phosphorylation of cTnl in Serine 22/23 as candidate biomarker of CHF.	[82]
SHR versus RHR and WKY	2D-DIGE/MALDI TOF	Comparison between two distinct models of heart hypertrophy: (i) 29 protein spots with differential expression among the three groups (18 proteins identified); (ii) ↓ GSTM2 and SCAD in RHR versus WKY; (iii) Distinct profile of GSTM2 and SCAD between SHR and RHR.	[49]
WKY	2-DE/MALDI TOF; Pharmacologic treatment	Effect of pharmacologic treatment over LVH regression: (i) 36 proteins altered in hypertensive heart; (ii) ↑ SCAD, NADH, enolase 1α, and aldehyde dehydrogenase; (iii) ↓ ETF-α, superoxide dismutase, and thiol-specific antioxidant.	[50]
Animal model lacking Kir6.2 ATP-sensitive K(+) (K(ATP)) channels	2-DE; LC-MS/MS; Orbitrap MS	Deficiency in myocardial K_{ATP} channels and hypertension pathophysiology: (i) 170 proteins with differential expression in response to K_{ATP} channel dysfunction; (ii) LDH, SCAD, pyruvate kinase, TPI, and CK.	[51–53]
Animal model and human	Transcriptome	Proteinases and the pathophysiology of hypertension: (i) Induction of cardiac hypertrophy by Ang II; (ii) Attenuation of cardiac hypertrophy by Ang II and RAS inhibition.	[41, 62–66]
dTGR and Sprague-Dawley rats	LC-MS/MS	Caloric restriction in dTGR over mitochondrial proteins: (i) 7 differential proteins after caloric restriction in Dtgr; (ii) 6 proteins unique to dTGR compared to caloric restricted dTGR and SD rats; (iii) ↓ 6 proteins (cytoskeletal and enzyme modulators) and ↑ oxidoreductase.	[69]
dTGR	Gas-chromatography TOF	Cardiac hypertrophy induced by Ang II: Modulation of >100 cardiac metabolites.	[70]
Aortic constriction in rodent model	Label-free LC-MS/MS	Pressure overload cardiac hypertrophy: (i) ↓ mitochondrial fatty acid oxidation proteins; (ii) ↑ pyruvate dehydrogenase subunits and TCA proteins.	[71]
Animal model and cell culture	2-DE; LC ESI-MS/MS	Cardiac hypertrophy induced by ET-1 and leukemic inhibitory factor exposure: (i) Differential proteome between ET-1 (concentric) and eccentric induced hypertrophy; (ii) ↑ αB-crystalline in nontreated cells; (iii) ↑ ANP upregulated in both cardiac hypertrophy models; (iv) ↑ desmin protein species.	[43, 79]

Table 1: Continued.

Experimental model	Experimental method	Main altered proteome	Reference
Animal model and cell culture	2-DE MS/MS	Cardiac hypertrophy induced by ISO: (i) 7 differential expressions in heart induced with ISO; (ii) ↓ MLC 2 and 3, desmin, prohibitin, FABP-H, and ATP-synthase 5β; (iii) ↑ HSP60, 70, and D1.	[80, 81]

ANP: atrial natriuretic polypeptide; CHF: chronic heart failure; CK: creatine kinase; cTnl: cardiac troponin; dTGR: double transgenic rats harbouring human renin and angiotensin genes; ET-1: endothelin-1; ETF-α: electron transfer flavoproteins-α; FABP-H: heart fatty acid binding protein; GSTM2: glutathione-S-transferase; Hadha: mitochondrial trifunctional enzyme alpha subunit; HSP: heat shock proteins 60, 70, and D1; ISO: isoproterenol; LDH: lactate dehydrogenase; LVH: left ventricle hypertrophy; MLC 2 and 3: myosin light chain 2 and 3; NDUFA10: NADH dehydrogenase 1 alpha subcomplex 10; RHR: animal model of secondary hypertension performed by clipping renal arteries; SCAD: Short-chain acyl-CoA dehydrogenase; SHR: spontaneously hypertensive rat; TCA: tricarboxylic cycle; TPI: triosephosphate isomerase; WKY: Wistar-Kyoto; β-MHC: β-myosin heavy chain.

hypoxia developed hypertension, while 2-DE analysis indicated changes in protein involved in the renal kallikrein system (kallistatin and A1AT) and regulation of vascular hypertrophy. In contrast, rats submitted to sustained hypoxia presented an upregulation of b2-bradykinin receptor and elevated kallikrein levels with normalized levels of blood pressure not developing hypertension. These data suggest that these alterations in the renal proteome in response to sustained hypoxia are related to a compensatory effect in vasodilation and vascular remodelling in order to prevent the development of hypertension. In another study using classic 2-DE analysis, Pinet et al. [88], using a two-kidney, one-clip method in Goldblatt rat model or renovascular hypertension, found that troponin T decreased in renal arterioles from the clipped kidney, indicating this protein as a possible biomarker in the pathophysiology of renovascular hypertrophy.

The urinary proteome has also attracted much interest in hypertension research, because clinical proteomics aims to detect possible biomarkers for left ventricle diastolic dysfunction and diastolic heart failure (associated with hypertension). Moreover, the urinary proteome may represent an advance in the early diagnosis of hypertension. In this context, Kuznetsova et al. [83] used capillary electrophoresis coupled with mass spectrometry (CE-MS) to screen and identify peptides and polypeptides (collagen polypeptides) that might be associated with the early stage of left ventricle dysfunction in hypertensive patients. After initially identifying 85 potential biomarkers ($P < 0.033$), the authors identified three polypeptides (collagen alpha-1(V), WW domain-binding protein 1,1 and isoform 1 of collagen alpha-1) that were significantly downregulated in patients with LV dysfunction compared to control patients. However, the authors also stated the need for larger cohort studies to better establish the accuracy of using the urinary proteome to identify new biomarkers in LV dysfunction. The urinary proteome has also been used to identify conditions associated with hypertension, such as preeclampsia renal injury [89–91] and other pathologies associated with hypertension such as diabetes [92].

In addition, the relationship between hypertension and arterial thrombosis was investigated by analysing the platelet proteome by 2-DE [93] in two distinct rodent models of induced hypertension (cyp1a1ren-2 transgenic rats fed with indole-3-carbinol and Fischer 344 rats induced with subcutaneous infusion of angiotensin II). In this study, 45 proteins spots were shown to be altered during hypertension induction in both animal models, and the expression of all protein spots was reversed after 10 days of blood pressure normalization. Moreover, the authors identified by mass spectrometry 38 spots that were assigned to 20 proteins (mainly protein fragments), which indicate that hypertension induced by angiotensin II may be associated with protein degradation in platelets. The reversible aspect in this proteome study has led to the prospect of identifying and developing possible novel biomarkers.

6. Does Exercise Extenuate Cardiac Pathological Hypertrophy?

Cardiomyocyte plasticity plays an important role in heart adaptation and maladaptation to external stimuli such as pregnancy, exercise, chronic pathology, and genetic disorders. As mentioned during this review, cardiac remodelling is a complex phenotype modification resulting from adverse external and intrinsic stimulus followed by alternative inner cell signalling, gene regulation, and cardiac proteome modulation [11, 94, 95]. In this context, physiologic and pathologic hypertrophy display a distinct molecular mechanism, also confirmed by proteomic data [11, 94]. In the previous section, several proteins related to metabolism, myocyte contraction, and stress response were shown to be altered in pathological hypertrophy, especially in LV. Thus, these proteome modulations were associated with the altered metabolism, fibrosis, and contractive dysfunction seen in hypertensive hearts [49, 50]. Lastly, pathological hypertrophy is characterized as an irreversible process.

Contrarily, physiologic cardiac hypertrophy in response to pregnancy and chronic exercise is a reversible process and associated with improvement in cardiac function and increased heart resistance to ischemic insult [11]. Exercise stimuli have been extensively shown to modulate the heart proteome [94, 96–102] which is normally followed by an improvement in aerobic capacity [98]. Furthermore, improved aerobic capacity is an independent factor for health status, being also inversely correlated with cardiovascular diseases [103], with exercise being a strong factor for preventing and treating hypertension and associated pathologies

such as obesity and diabetes [104]. Moreover, exercise is a nonpharmacologic agent and the main choice for hypertension treatment among other cardiovascular diseases, such as heart failure and myocardial infarction [1].

The role of exercise stimulus in blood pressure (BP), endothelial function, and cardiac hypertrophy in the experimental model is still under debate [105], where exercise intensity seems to be a key factor in this process [106]. Research has shown that low exercise training attenuates systolic hypertension and improves mitochondrial status and contractile dysfunction, delaying heart failure in a hypertensive experimental model [16, 107]. While moderate exercise (70% of maximal running speed) did not affect BP, it did not worsen cardiac function in severe hypertensive rats induced by renal artery constriction (two-kidney, one-clip model) [18]. Moreover, exercised hypertensive rats (SHR) were shown to present reduced levels of BP compared to sedentary SHR, while exercised SHR and sedentary nonhypertensive rats (Wistar) presented a reduced aorta wall thickness compared to sedentary SHR [108]. Exercise has also been shown to correct abnormal Ca^{2+} handling in heart failure rats [14], attenuate systolic dysfunction, and improve bad phosphorilation (e.g., pro-apoptotic molecule) in the early stage of hypertension, independent of relieving apoptosis [107]. Exercise training was also shown to superimpose hypertension impacts on LV remodelling, increasing cardiomyocyte length and width to a greater degree than in nontrained SHR, also attenuating apoptosis [109]. Cardiac mitochondrial apoptotic signalling was also shown to be reduced by aerobic exercise in an obese animal model with prehypertensive BP status [110]. On the other hand, it has been shown that high exercise intensity may be considered a risk factor in the hypertensive phenotype rather than a therapeutic factor, since this intensity was shown to accelerate hypertensive effects and improved fibrosis in SHR [111]. Despite these data from the literature, there is still a need for more proteomic data concerning the effect of exercise on the pathological heart to better understand the effects of exercise on pathological hypertrophy induced by hypertension.

Concerning the metabolism status, it has been demonstrated that rodents with natural inborn low aerobic capacity harbour an altered and perturbed energy metabolism and an enhanced oxidative stress in heart proteome [112]. Contrary to the metabolic dysfunction (e.g., reduction in FFA oxidation) seen in hypertensive hearts, endurance exercise is associated with improvements in cardiac metabolic enzymes, especially in fatty acid oxidation as reviewed by Burniston and Hoffman [94].

In this regard, the expression of several metabolic enzymes (short-chain acyl-CoA dehydrogenase and enzymes from the β-oxidation TCA cycle) from LV was shown to be altered after high intensity swimming [102]. Swimming training also led to cardiac hypertrophy in nonpathological rats. Further research in LV proteome showed that moderate treadmill running led to diverse alteration in the contractile, stress-related, and metabolic function of cardiac proteins, where heart fatty acid binding proteins (HFABP), thioesterase-1, and short-chain acyl-CoA dehydrogenase were upregulated [98]. Moreover, one single bout of high intensity swimming at moderate and high intensity was also shown to modulate LV proteins from obese (ob/ob) and control nonobese mice (ob/OB) [100]. However, in this study, HFABP was downregulated after high intensity exercise in nonobese mice but not in obese mice. Moreover, aspartate aminotransferase, an analogue of plasma membrane fatty acid transporter (FABPpm), was also upregulated in nonobese mice, possibly indicating an acute uptake of long-chain fatty acids. In this study, mitochondrial aconitase was downregulated in both rodent phenotypes, while HFABP was downregulated only in obese mice. In a recent study, the LV proteome from Wistar rats was shown to be modulated, following different swimming exercise intensities adjusted according to each animal's body weight [101]. Moderate and high intensity resulted in the upregulation of contractive proteins, mainly α-MHC (alpha-myosin heavy chain) and troponin accompanied by cellular injury in the high intensity group. The metabolic enzyme, NADH dehydrogenase, was also differentially expressed in response to high exercise intensity. Although high intensity was associated with greater proteome changes, this intensity was associated with cardiac cell damage compared to low and moderate intensities. Alteration in contractile, metabolic, and mitochondrial enzymes induced by endurance exercise occurred in an opposite way from the changes seen following pressure overload pathological hypertrophy and heart failure [71].

In an ischemia/reperfusion experiment, exercise training was shown to alter cardiac mitochondrial proteins and protect the heart against IR-induced myocardial damage, also by presenting an antiapoptotic effect [99]. In research using isobaric tags for relative and absolute proteome quantitation (iTRAQ), authors identified 222 mitochondrial proteins, where 13 were significantly altered by endurance training (8 upregulated and 5 downregulated). Moreover, downregulation of mitochondrial proteins, MAO-A (monoamine oxidade) and PRDXIII were identified as novel potential candidates of exercise-induced cardioprotection since they play a prominent role in oxidative stress and apoptosis, with MAO-A being associated with pressure overload pathology hypertrophy and heart failure [113, 114]. Moreover, relative and absolute proteome quantitation have significantly improved proteomic investigation in several areas including cardiovascular research [115–117]. More recently, endurance exercise was shown to play a positive role in cardiac function after myocardial infarction [96]. Two-DE analysis revealed that exercise training induced the upregulation of glutathione peroxidase-1 and manganese superoxidase dismutase, with both being related with antioxidative activity induced by exercise [118].

Lastly, heat shock protein 20 is a widely researched chaperone due to its role in cardioprotection [119, 120]. Boluyt et al. [97], in the first study involving exercise and the cardiac proteome, demonstrated that six weeks of endurance training led to adaptive cardiac hypertrophy and significantly altered 26 protein spots in LV, where 12 spots, including the HSP20, were exclusive to trained rats. Authors also showed that the expression of shp20 only followed exercise training rather than a single bout of exercise. Furthermore, shp20 was also shown to be upregulated in Wistar rats following moderate

Table 2: Challenges and future perspectives in cardiac proteome in hypertension research.

Challenges in cardiac proteomic and hypertension research:
(i) integration of "omics" tools as a multiple strategy;
(ii) MS-based proteomics coupled with NGS approach;
(iii) proteomic and genomic large-scale studies in hypertension development and treatment;
(iv) identification of posttranslational polymorphism and genetic factors;
(v) identification of novel differential molecular signalling and expression between physiologic and pathologic cardiac hypertrophy;
(vi) identification of novel hypertension biomarkers in blood samples.
Future direction in cardiac proteome and hypertension research:
(i) novel studies cross talking proteomic and genomic data;
(ii) improvement in gene expression quantitation and transcriptome data;
(iii) identification of novel pharmacologic targets and nonpharmacologic strategies in hypertension attenuation;
(iv) novel drug design and texting in cellular and experimental hypertensive models;
(v) investigation of exercise and other alternative strategies in hypertension attenuation.

NGS: next generation sequencing.

exercise endurance (75% of $VO_{2\,max}$), compared to sedentary animals [98]. The proposed exercise program was shown to increase cardiac mass (11%) and to improve animals' aerobic capacity ($VO_{2\,max}$ increase by 23%). In this study, MS/MSA revealed that exercise-induced shp20 is phosphorylated at Serine 16 in a few hours after exercise. Again, protein phosphorylation may be associated with a cardioprotection process, since the blockade of HSP20phosphorylation is shown to enhance ischemia/reperfusion injury [121].

Despite the scarcity of proteomic research performed with exercise and heart tissue, the present data indicated that the altered proteome is mostly associated with cardioprotective aspects such as contractile and metabolic improvement and physiologic cardiac hypertrophy. Moreover, the degree of cardiovascular adaptation to exercise is intensity dependent, where, as previously shown, high intensity exercise may enhance hypertensive stimulus [111] and be associated with cardiac damage [101]. Thus, it is suggested that more research should be performed, taking into account the effect of different types and intensities of exercise on the heart proteome.

7. Conclusions and Prospects

The various advances in high-throughput platforms have led to multianalysis of genes, proteins, and other molecular components that may be involved in hypertension pathogenesis and pathophysiology. Therefore, despite progress in proteomic research, the multifactor aspect of hypertension still needs to be explored by a multiplex strategy, which certainly involves a number of other "omics" tools and analysis strategies such as those seen in systems biology. In this view novel techniques in addition to classical proteomics tools including mass spectrometry- (MS-) based proteomics, posttranslational modifications detections, and next-generation sequencing (NGS), which are fast maturing procedures, are enabling comprehensive measurements of gene products at a system of hypertension pathogenesis and pathophysiology level [122]. Although MS and NGS are extremely complementary, they are still rarely applied and integrated in large-scale studies including exercise and hearth pathology. Nevertheless, all those techniques must also apply together in order to shed some light on those important and complex systems. Technological advances in both the proteomics and transcriptomics community also may offer the capability to distinguish genetic and post-transcriptional polymorphisms at the proteome level. These advances also allow improved gene expression quantitation, which is restricted by the imprecise proxy of transcriptome data alone. In summary authors believe that synergistic utilization of multiple techniques including genomic, transcriptomic, and proteomic technologies will significantly improve information, enhancing proteogenomics to a top level in exercise and hypertension studies. The main challenges in cardiac proteomic and hypertensive research and the future directions on this field are presented in Table 2. Such actions are remarkable challenges for the next years and could, in our opinion, clearly contribute to development of cardiac and hypertension proteomics.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Analysis of Pericardial Effusion from Idiopathic Pericarditis Patients by Two-Dimensional Gel Electrophoresis

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Pericardial fluid (PF) is often considered to be reflection of the serum by which information regarding the physiological status of the heart can be obtained. Some local and systemic disorders may perturb the balance between synthesis and discharge of PF and may cause its aberrant accumulation in the pericardial cavity as pericardial effusion (PE). PE may then lead to an increased intrapericardial pressure from which the heart function is undesirably affected. For some cases, the causes for the perturbance of fluid balance are well understood, but in some other cases, they are not apparent. It may, thus, be helpful to understand the molecular mechanisms behind this troublesome condition to elucidate a clinical approach for therapeutic uses. In this study, protein profiles of PEs from idiopathic pericarditis patients were analyzed. Control samples from patients undergoing elective cardiac surgery (ECS) were included for comparison. In addition to high abundant serum-originated proteins that may not hold significance for understanding the molecular mechanisms behind this disease, omentin-1 was identified and its level was higher for more than two-fold in PE of IP patients. Increased levels of omentin-1 in PE may open a way for understanding the molecular mechanisms behind idiopathic pericarditis (IP).

1. Introduction

The space between the parietal and visceral layers of the pericardium contains small amount of fluid called PF. PF has a discernible lubricant function [1, 2]. The composition of normal PF can be described as an ultrafiltrate of plasma except with low protein content [3–5]. Some systemic and local disorders such as coronary artery diseases, malignant diseases, connective tissue disorders, idiopathic causes, inflammation, tumors, or hemorrhage may disturb the balance between formation and removal of PF and cause its accumulation as PE [5, 6].

The causes of pathological PE are not always clear and the etiology is unknown in more than 50% of the cases [7–9]. A systematic approach for diagnostic testing based on standardized practice guidelines has been proposed [10]. A diagnostic pericardiocentesis and/or pericardial biopsy are/is recommended for large/recurrent effusions if conventional tests remain inconclusive. Unfortunately, analysis of the biochemical and cell-count composition of the pericardial fluid is generally not helpful for the diagnosis of most pericardial effusions [11]. Therefore, a large proportion of the cases are labeled as idiopathic pericarditis (mean: 26.1%), followed by neoplastic diseases (mean: 25.6%) and iatrogenic pericarditis (mean: 16.3%) [9].

By using biochemical approaches, the presence of putative biomarkers like CRP was proposed in PE of pericarditis patients [8, 12, 13]. However, those biomarkers did not find place in clinical practice. Proteomic approaches may help to identify incipient biomarkers to fulfill the needs in cardiovascular diseases including pericarditis [14]. However, until recently an extensive study examining the potential

utilization of PF as a source of biomarkers was missing. Fortunately, recently published study reported an extensive list of low abundant proteins from PF and highlighted that, as a biochemical window of heart, PF proteome can be a good material for cardiovascular research [15]. In this study, we used two-dimensional gel electrophoresis (2D) to examine the protein profile of PE from IRP patients. The results showed that, unlike the control samples from ECS patients, omentin-1 can readily be detectable in 2D gels prepared from PE samples making it a putative marker for the disease.

2. Methods

The study was approved by the institutional review board and informed consents were obtained from all patients.

2.1. Sample Collection. A subxiphoid vertical incision was made under general anesthesia and pericardial cavity was entered. After opening a pericardial window, a pericardial biopsy was taken and drainage was performed. The pericardial fluid samples were subjected to biochemical, microbiological, and pathological examinations. Thoracic tomography and ultrasonography were performed to all patients for tumor detection. The study group was composed of seven IRP patients for whom no diagnosis was possible to explain the presence of PE. Blood-free PE samples were collected in sterile tubes without anticoagulant. Similarly, PF samples from ECS patients were collected to form a control group. After centrifugation at 3000 ×g for 10 min at 4°C, the supernatants were collected and aliquoted into Lo-Bind storage tubes (Eppendorf Inc., USA) and stored at −80°C until use. The protein concentrations were measured with RC-DC protein assay (Bio-Rad, USA).

2.2. MicroRotofor Fractionation. One mL of each sample was desalted through a 10 DG column (Bio-Rad, USA) and buffer exchange was performed with 10 mM Tris.Cl, pH 6.8. After combining protein containing fractions that were eluted from 10 DG column, three mL of the combined fractions was mixed with 40% ampholyte (pH 3–10) to obtain 2% final ampholyte concentration. The sample was then loaded to a MicroRotofor unit (Bio-Rad, USA) and focused for 3 hr at 1 W. At the end of the focusing period, ten fractions from each sample were collected and 5 μL of each fraction was subjected to SDS-PAGE for analysis of fractionation efficiency. To remove the excess ampholyte that originated from MicroRotofor fractionation, the fractions were dialyzed against 100-fold diluted 2D sample buffer by using a Slide-A-Lyzer dialysis unit with a MW cut-off limit of 2000 (Pierce, USA) and carefully recovered without a significant protein loss.

2.3. Two-Dimensional Gel Electrophoresis (2DE). Protein fraction number four of each sample obtained from MicroRotofor was subjected to 2DE analysis. Eighty μg of protein was actively (50 V) loaded to IPG strips (11 cm, pH 5–8, Bio-Rad) at 20°C for 16 hr and then run through a stepwise incremental voltage program (250 V for 20 min (linear), 4000 V for 2 hr (linear), and 10000 V/hr (rapid)) by using Protean IEF system (Bio-Rad, USA). The strips were then subjected to a two-step equilibration in equilibration buffers containing 6 M urea, 2% SDS, 0.375 M Tris.Cl pH 8.8, 20% glycerol and 2% DTT for the first step and 6 M urea, 2% SDS, 0.375 M Tris.Cl pH 8.8, 20% glycerol and 2.5% iodoacetamide for the second step. The strips were then transferred onto the second-dimension SDS-PAGE equipment and proteins were separated on 12% polyacrylamide gels. Protein spots were visualized by using SyproRuby fluorescent stain.

2.4. Image Acquisition and Analysis. Gel images were taken with an imaging system (VersaDoc4000MP, Bio-Rad, USA) and analyzed by using PDQuest Advanced 2D-image analysis software (Bio-Rad, USA). The quantity of each spot was normalized using local regression model. Based on average spot volume ratio, spots whose relative expression levels were changed at least 2-fold (increase or decrease) among the compared groups were considered to be significant. Statistical significance was assessed by using student's t-test ($P < 0.01$). Protein spots that displayed statistically significant regulation were cut by using automated EXQuest Spot Cutter (Bio-Rad) and deposited in a 96-well plate for in gel-tryptic digestion.

2.5. Tryptic In-Gel Digestion and MALDI-TOF/TOF Analysis. MALDI-TOF MS and TOF/TOF tandem MS/MS were performed by Applied Biomics (http://www.applied-biomics.com/index.html; Hayward, CA, USA) using an AB SCIEX TOF/TOF 5800 System (AB SCIEX). The resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix Science Inc.) to search the National Center for Biotechnology. Additional information for the MS/MS database search parameters and protein identification can be found in Supplementary Tables 1, 2, and 3, respectively (see Supplementary Materials available online at http://dx.doi.org/10.1155/2014/942718).

2.6. Western Blot Analysis. Equal volume of PE/PF from fraction four of each fractioned sample was mixed to form protein pools of study and control groups, respectively. After SDS-PAGE electrophoresis, proteins were transferred to a nitrocellulose membrane from an SDS-PAGE gel using a semidry transfer apparatus following the instructions provided by the manufacturer (TurboBlot, Bio-Rad, USA). The membrane was then probed with an anti-omentin-1 monoclonal antibody (Clontech, USA) using the chemiluminescent detection system (GE Healthcare, USA). The images were recorded with VersaDoc MP4000 (Bio-Rad, USA) and a set of prestained protein markers (Fermentas, USA) was used to assess the size of the signal (~40 kD) generated in western blots. For the purpose of spot analysis, ImageJ, freely available software, was used. The integrated density of each protein band was measured by outlining them and using the analyze/measure command.

Table 1: Demographic features and biochemical test results for the RIP and ECS patients.

Demographic features	Pericardial effusion							Elected cardiac surgery				
	1	2	3	4	5	6	7	1	2	3	4	5
Sex (male/female)	F	F	M	M	F	M	F	M	M	F	F	M
Age (year)	58	45	63	70	59	42	69	41	63	53	65	72
Hypertension (+/−)	+	−	+	+	+	−	−	−	−	+	+	+
Diabetes mellitus (+/−)	−	−	−	−	−	−	−	−	−	+	−	+
Myocardial infarction (+/−)	−	−	−	−	−	−	−	−	+	+	−	−
Ejection fraction (%)	66	60	65	68	88	67	56	45	40	30	60	78
Biochemical test results												
BUN (mg/dL)	13.1	10	15	9	14	18	13	13	17	20	16.5	21
Creatinine (mg/dL)	0.65	0.66	0.5	0.8	0.9	0.71	0.7	0.8	0.9	0.6	0.79	1.02
GOT (U/L)	18	11	28	16	17	25	60	18	53	23	28	24
GPT (U/L)	16	15	18	11	12	38	14	22	37	21	18	18
Albumin (g/dL)	4.41	3.9	3.1	3.42	3.4	3.45	3.6	4.4	4	3.5	3.6	3.9
LDH (U/L)	216	215	251	205	230	169	334	237	210	183	178	192
TP (g/dL)	7.26	7.1	6.6	6.5	5.9	6.3	8.2	7.1	7.3	6.8	7	7.5
LDH (PE) (U/L)	95	130	141	138	167	102	147					
TP (PE) (g/dL)	2.2	5.1	3.6	3.4	2.8	3.2	3.6					
CRP (mg/L)	0.231	0.293	0.6	0.23	2.03	1.7	0.6	0.3	0.2	0.76	1.26	0.29
ADA (u/L)	9.20	22	15	24	38	18	23					
TSH (U1u/mL)	0.994	0.665	1.203	1.27	0.22	1.69	4.4	0.8	0.768	0.63	0.71	1.11

Table 2: Tryptic peptides of omentin-1 identified by MALDI-TOF/TOF analysis.

Calculated mass	Observed mass	±Da	±ppm	Start sequence	End sequence	Sequence
2059.9792	2059.8721	−0.1071	−52	43	59	EIKDECPSAFDGLYFLR
1292.5906	1292.6897	0.0991	77	219	229	TASYYSPYGQR
1201.6	1201.6957	0.0957	80	230	239	EFTAGFVQFR
1424.7783	1424.8894	0.1111	78	302	313	EITEAAVLLFYR

3. Results

Clinical features of seven patients and five controls were presented at Table 1. There was no significant difference among the IP and ECS patients in their demographic properties and standard serum biochemical test results. All of the studied samples were transudative. There was no history of trauma and hemorrhage in any of the subjects. Microbiological examination of effusions and pericardial tissue biopsies indicated no definite bacterial or fungal infections. Bacterial staining tests for acid-alcohol fast bacteria and mycobacteria were negative, and serum adenosine deaminase (ADA) screening remained negative for tuberculosis. Serologic screening for autoantibodies indicated no abnormality as well. Cytological and pathological examination of tissue samples revealed the absence of malign cells, and this finding was also supported by the low levels of serum tumor markers (Ca-125, Ca 15-3, Ca 19-9, CEA and AFP) (data not shown).

When samples were subjected to SDS-PAGE without prefractionation, high abundant proteins were apparent and needed to be reduced to enrich low abundant proteins. A Isoelectric Point (pI)-based fractionation approach was used to allow detection of low abundant proteins. SDS-PAGE analysis of each fraction revealed that fractionation enriched some of the minor proteins by placing majority of the albumin into a single fraction (Figure 1(a)). 2D analysis of each fraction confirmed this finding. Fraction number four contained protein spots that were otherwise not detectable on 2D gels prepared from unfractionated samples. (Figures 1(b) and 1(c)). MALDI-TOF/TOF analysis of some of the selected spots indicated the presence of abundant proteins such as albumin or albumin in a complex with myristic and triiodobenzoic acids (Pro2675), immunoglobulin, and hemopexin (Figure 1(c), Supplementary Table 2). These spots matched with the plasma 2DE-map [16].

Among the identified spots, peptides belonging to omentin-1 (alternative name: intelectin-1) were readily detectable in PE samples (Table 2, Supplementary Table 3). MALDI-TOF/TOF analysis identified four peptides out of 26 possible tryptic peptides which accounted for the recovery of 16% of whole omentin-1 sequence with high confidence interval (Figure 2). When WB analysis was performed from

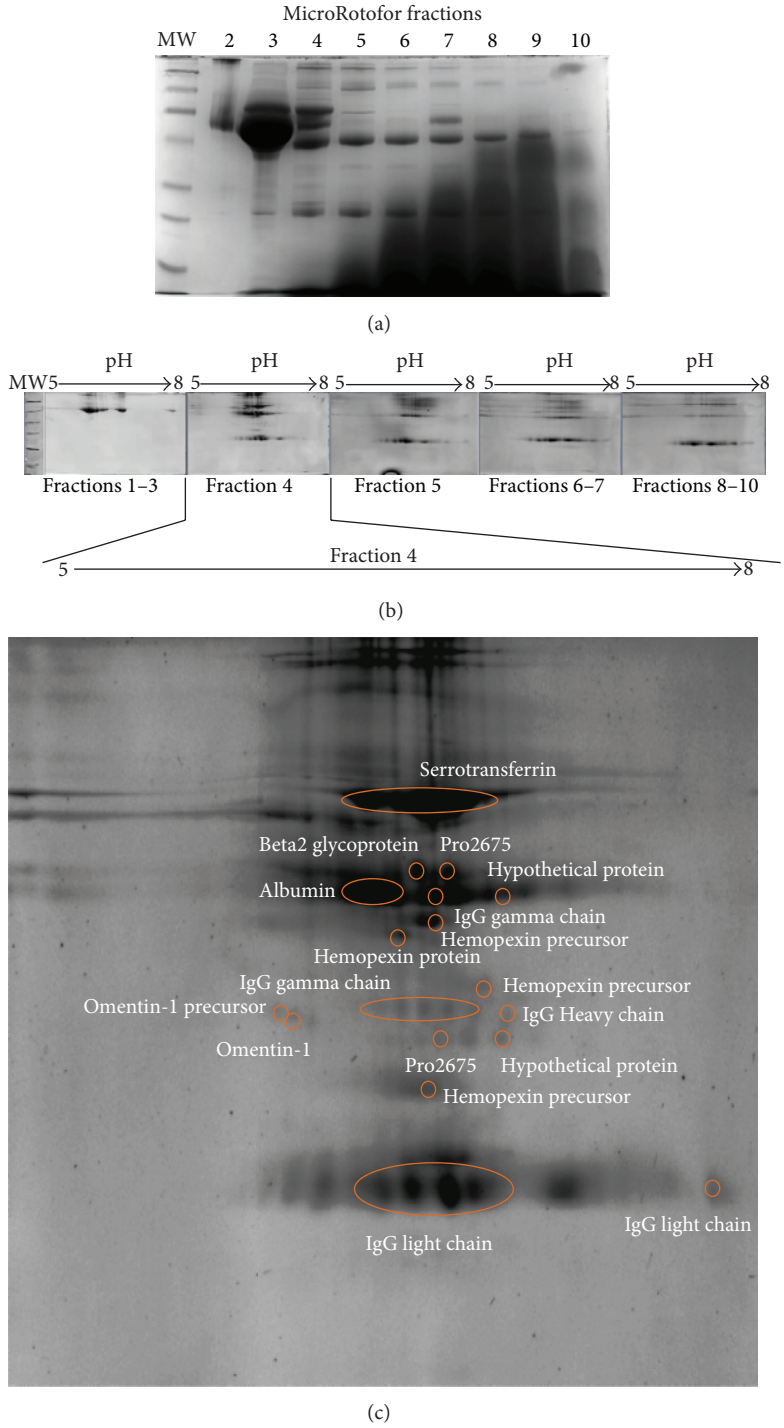

Figure 1: (a) SDS-PAGE analysis of MicroRotofor fractions of a PE after 3 hr. of focusing at 1 W. Notice that majority of the albumin was collected in fraction 3. The shadowy darkness at an increasing pattern was due to ampholyte (pH 3–10) that was added to the protein mixture at a concentration of 2% before focusing. (b) 2D analysis of MicroRotofor fractions. Fraction 4 was the only fraction that contained detectable level of omentin-1. (c) Positions of the protein spots that were identified in fraction 4. Despite the extensive prefractionation, high abundant proteins were still dominating the gel.

protein pools of the study and control groups, omentin-1 was found to be present more in the pooled sample from the study group. Measurement of band intensities revealed more than 2-fold increase in omentin-1 levels (Figure 3).

4. Discussion

As a biochemical window of heart, PF may hold the potential as a biomarker to assist in diagnosis of various heart diseases. Because biomarkers are mostly proteins, studying protein

```
              |           |           |  |  |             |
MNQLSFLLFLIATTRGWSTDEANTYFKEWTCSSSPSLPRSCKEIKDECPSAFDGLYFLRT

                                  | |     |       |
ENGVIYQTFCDMTSGGGGWTLVASVHENDMRGKCTVGDRWSSQQGSKADYPEGDGNWANY

               |          |          |      |     | |
NTFGSAEAATSDDYKNPGYYDIQAKDLGIWHVPNKSPMQHWRNSSLLRYRTDTGFLQTLG

        |   |    |                     |          |         |
HNLFGIYQKYPVKYGEGKCWTDNGPVIPVVYDFGDAQKTASYYSPYGQREFTAGFVQFRV

    |         |
FNNERAANALCAGMRVTGCNTEHHCIGGGGYFPEASPQQCGDFSGFDWSGYGTHVGYSSS

|
REITEAAVLLFYR
```

Figure 2: Complete amino acid sequence of omentin-1. Vertical lines represent possible tryptic digestion points and underlined peptides are the ones detected by MALDI-TOF/TOF analysis.

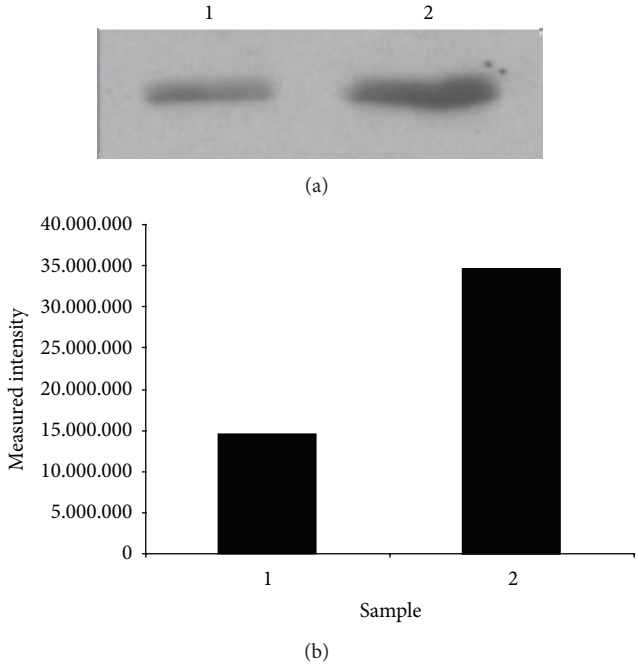

Figure 3: (a) Western blot analysis of the pooled PF samples from ECS patients and PE samples from IP patients. The 4th fraction after MicroRotofor fractionation was pooled and used for analysis. (b) Graphical presentation of the band intensities measured with ImageJ.

profile of PF may hold great importance. However, there have been limited efforts to perform an in-depth analysis of the PF proteome. This may be due to the fact that PF is a hard to reach material to reach and its collection requires invasive procedures. In a previous study, Liu et al. (2008) described tuberculosis related proteins in PE samples obtained from tuberculosis patients with heart failure and identified a number of differentially expressed tuberculosis-related proteins [17]. However, their study failed to describe a cardiac related protein as a biomarker. In a recent study, Xiang et al. (2013) described proteomic profiling of PF and identified over 1000 nonredundant proteins to generate the first comprehensive PF proteome (Xiang et al., 2013). However, their study excluded the patients with evidence or history of cardiac or pericardial diseases and thus is only descriptive in nature. In this study, we used 2DE approach to examine protein profiles of PE samples from IP patients and compared them with protein profiles of PF samples of patients undergoing ECS hoping to find a potential biomarker for differentiation of IP.

The only protein that created interest in terms of its elevated presence in the IP patients was omentin-1 (NCBI accession # 119573073). Omentin-1 is a relatively recently identified novel adipocytokine whose involvement in obesity, insulin resistance, and diabetes is recognized [18–21]. In addition, omentin-1 levels are altered in chronic inflammatory conditions particularly in autoimmune diseases [22]. In fact, omentin-1 was proposed to be a potential biomarker in synovial fluid for reflecting the degenerative process in osteoarthritis [23]. The involvement of omentin-1 in cancer was also proposed based on the finding that omentin-1 gene overexpression was 139-fold higher in malignant pleural mesothelioma cells [24]. Circulating omentin-1 levels was also proposed to be an independent marker for arterial stiffing in patients with type-2 diabetes [25, 26]. In some studies, omentin-1 levels were measured and associated with cardiovascular diseases [27, 28].

Although omentin-1 is a main indicator of inflammation, these and similar studies clearly demonstrated the pleiotropic nature of omentin-1 which appears to have a role in regulating various metabolic events in our bodies [29]. Therefore, a detailed understanding of themolecular mechanisms by which these regulations occur is needed. In this study, we reported the elevated levels of omentin-1 in PE of IP patients and proposed that omentin-1 might be an indicator of the disturbed pericardial balance.

The etiology and pathogenesis of IP remain controversial standing like a bridge that crosses infectious, autoimmune, and autoinflammatory pathways [29]. Microorganisms such as viruses, bacteria, and fungi can cause the pericardial infections. The most common viral pathogen is known to be coxsackie virus and echovirus. Other common agents are

cytomegalovirus, herpes virus, and HIV [30, 31]. However, details of pericarditis caused by other infectious agents are not yet known. Therefore, various treatment strategies are employed. Nonsteroidal anti-inflammatory drugs must be used at recommended dosages to resolve the symptoms so that normalization of C-reactive protein and erythrocyte sedimentation rate are reached [31]. Corticosteroids should be used rarely, at low doses, with an extremely low tapering and with osteoporosis prevention [32]. Colchicine leads to a clinically important and statistically significant benefit, reducing recurrences. Surgical treatment of pericardial pathologies is reserved for symptomatic patients. The choice of procedure could be partial or complete pericardiectomy, pericardioplasty or actualincision. Longitudinal extension of the defect to relieve tension on critical structus performed [33].

5. Conclusions

In conclusion, the value of pericardial fluid as a biomarker source for the detection of cardiovascular diseases cannot be underestimated. When the dynamic nature of pericardial fluid—its continuous reproduction and the drainage—is considered, its importance becomes more apparent in biomarker discovery research. In this study, the proteomes of PEs were examined to some extent.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Cardiac Phosphoproteomics during Remote Ischemic Preconditioning: A Role for the Sarcomeric Z-Disk Proteins

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Remote ischemic preconditioning (RIPC) induced by brief ischemia/reperfusion cycles of remote organ (e.g., limb) is cardioprotective. The myocardial cellular changes during RIPC responsible for this phenomenon are not currently known. The aim of this work was to identify the activation by phosphorylation of cardiac proteins following RIPC. To achieve our aim we used isobaric tandem mass tagging (TMT) and reverse phase nanoliquid chromatography tandem spectrometry using a Linear Trap Quadropole (LTQ) Orbitrap Velos mass spectrometer. Male C57/Bl6 mice were anesthetized by an intraperitoneal injection of Tribromoethanol. A cuff was placed around the hind limb and inflated at 200 mmHg to prevent blood flow as confirmed by Laser Doppler Flowmetry. RIPC was induced by 4 cycles of 5 min of limb ischemia followed by 5 min of reperfusion. Hearts were extracted for phosphoproteomics. We identified approximately 30 phosphoproteins that were differentially expressed in response to RIPC protocol. The levels of several phosphoproteins in the Z-disk of the sarcomere including phospho-myozenin-2 were significantly higher than control. This study describes and validates a novel approach to monitor the changes in the cardiac phosphoproteome following the cardioprotective intervention of RIPC and prior to index ischemia. The increased level of phosphorylated sarcomeric proteins suggests they may have a role in cardiac signaling during RIPC.

1. Introduction

Remote ischemic preconditioning (RIPC) is a powerful protective phenomenon in which brief ischemic periods of a remote organ (e.g., arm or leg) confers protection of another organ (e.g., heart) against a sustained ischemia-reperfusion (I/R) insult (reviewed by [1]). Clinical benefits of RIPC have been demonstrated in patients undergoing primary percutaneous coronary intervention [2–6]. The benefits have also been demonstrated in both pediatric [7, 8] and adult open heart surgery [9–14]. Meta-analysis further confirmed the cardioprotection by RIPC in adult cardiac surgery [15] and its potential clinical application is expected to be excellent [16]. However, the mechanism(s) underlying this intervention and how a preconditioning stimulus in a limb confers protection to the patients' heart is essential for maximizing the beneficial effects of RIPC. Essentially the preconditioned organ transmits a signal to the heart which in turn triggers changes in the myocardium that eventually results in protection. Several mechanisms have been proposed that are based on experimental models (reviewed by [1, 17]). For example it has been suggested that the contact between the remote preconditioned organ/tissue and the heart could include humoral [18–20], neural factors [20, 21], and systemic changes. It is suggested that protection by RIPC is triggered by release of metabolites (e.g., adenosine, bradykinin, and opioids) from the remotely preconditioned organ [22–26]. In fact recent work has shown that adenosine A1 receptors are directly involved in RIPC [27]. However, little is known about the changes in target tissue (myocardium) as a result of RIPC and *prior* to I/R. Most studies have largely reported changes in signaling pathways after reperfusion. These include the activation of intracellular kinases such as PKCε [28–30], activation of the reperfusion injury salvage kinase (RISK)

pathway and the survivor activating factor enhancement (SAFE) pathway in early reperfusion [31]. Several of these pathways are similar to what happens in ischemic preconditioning. In contrast, little is known about the activation of signaling pathways immediately following RIPC and prior to I/R. It has been recently proposed that phosphorylation of key proteins are essential for RIPC as hypoxic hearts of pediatric patients do not result in further increase in phosphorylation of key signaling proteins and thus were not protected by RIPC [32]. In order to highlight the importance of phosphorylation in RIPC, we used our recently characterized RIPC mouse model [33] to monitor the changes in the activation (phosphorylation) of cardiac proteins following RIPC. Therefore we studied the cardiac phosphoproteome in both RIPC and in control hearts using Tandem Mass Tagging (TMT). TMT is an isobaric mass tagging approach commonly used for quantitative proteomics which allows the comparison of up to six different samples in a single experiment [29]. With this approach, samples are labelled after extraction, making it applicable to the comparison of nonculturable samples unlike the more traditional metabolic labelling approaches such as stable isotope labelling with amino acids in cell culture (SILAC). Labelling is performed at the peptide level using a set of six amine-specific isobaric tags which covalently attach to the peptide N-terminus (and the amino group of lysine residues), thereby labelling all peptides in a given sample. Each isobaric tag consists of a unique reporter group (of *m/z* 126, 127, 128, 129, 130 or 131) and a mass normaliser which ensures that the overall mass of each tag is the same (see Figure 1 in Methods). Thus differentially labelled peptides are identical in mass (and therefore indistinguishable) at the Mass spectrometry MS1 level. However, during the liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) analysis, fragmentation of each peptide not only generates an MS/MS spectra from which the amino acid sequence of the peptide is determined, but also results in cleavage and release of the reporter groups which appear as a cluster of ions at the low mass end of each MSMS spectra. The relative intensity of each reporter ion in that cluster is then used to give a measure of the relative abundance of that peptide between the six samples under comparison. We have combined this approach with the use of titanium dioxide (TiO_2) for phosphopeptide enrichment, thus allowing us to compare the relative levels of individual phosphopeptides between control and intervention (RIPC) samples.

2. Methods

2.1. Animals. Male C57/BL6 wild-type mice (25–28 weeks old, 26–33 g) were used for all experiments. All animals were purchased from B&K Universal. Animals were kept at the University of Bristol Veterinary School until used. Treatment of animals and all procedures were in accordance with Home office guidance (Scientific Procedures) Act of 1986.

2.2. RIPC Model. The characteristics of RIPC mouse model have been published elsewhere [33]. In brief, mice were anesthetized by an intraperitoneal injection (0.020 mL/g weight) of 2.5% tribromoethanol (Avertin) (Sigma-Aldrich, UK), allowed five minutes to become fully anesthetized (evidenced by lack of response to toe or tail pinch). A specially designed small pressure cuff (1.6 × 9 cm) (Hokanson, Inc.) was placed around the hind limb at the inguinal level. Blood flow in the hind limb was monitored using laser Doppler flowmetry (moorLDI2 imager) and was also confirmed by the change in the leg skin color. Body temperature was maintained at around 37°C using a heating pad.

2.3. RIPC Protocol. RIPC was induced by 4 cycles of 5 min of limb ischemia at 200 mmHg followed by 5 min of reperfusion ($n = 6$). Control group ($n = 6$) had a deflated cuff placed on the lower limb. At the end of the procedure (50 min), the animals were immediately terminated by cervical dislocation and the heart extracted and ventricular tissue snap frozen in liquid nitrogen and stored at −80°C. The maximum number of samples that can be analysed in a single tandem mass tagging experiment is six. Therefore, we carried out two separate runs to analyze the 12 samples. Each run (Series) involving six samples, included three control and three RIPC samples. Fold change between RIPC and control were calculated separately for each series.

2.4. Protein Extraction and Quantification. Each ventricular tissue was weighed and added to lysis buffer containing (1% Noidet P-40/IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS in 1x PBS) and one tablet of each proteases and phosphatases inhibitor cocktails at a ratio of 10 μL per mg of tissue. The mixture was kept on ice. The biopsies were then homogenized at high speed for 12 sec (twice) using Minilys tissue homogenizer (Bertin technologies, France) which involved the use of bead beating technology. The homogenate was left 30 minutes shaking on ice, at 4°C and then centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant was collected. Protein concentration in the supernatant was determined with the total protein kit, Micro Lowry (Sigma, UK). Bovine serum albumin (BSA) at a stock concentration of 5 mg/mL was used to obtain a standard curve. For each sample, 400 μL of Lowry reagent was added and incubated at room temperature for 30 min, followed by adding 200 μL of Folin-Ciocalteu reagent incubated for 30 min. The absorbance was measured at 750 nm using spectrophotometer (Jenway7305, UK). Protein concentration in samples was determined by interpolating the densities from the standard reference curve.

2.5. Immunoblotting. Cardiac samples from Series 1 were used for both western blotting and phosphorproteomics (see below). Reducing sample buffer (5x, 4% SDS v/v, 0.1 M Tris-HCl pH 6.8, 10% mercaptoethanol, 25% glycerol) was added to the protein samples and was heated at 95°C for 5 min. In contrast proteins extracted for western blot to measure the phosphorylated phospholamban (PLB) were not boiled as per the manufacturer's protocol (Abcam, see below). 20 μg/mL of total protein were separated on 4–20% gradient gel (Mini-ProteanTGX, Bio-Rad, UK) and revealed using Western blotting with antibodies (diluted 1 : 2000) against P-PLB (Abcam

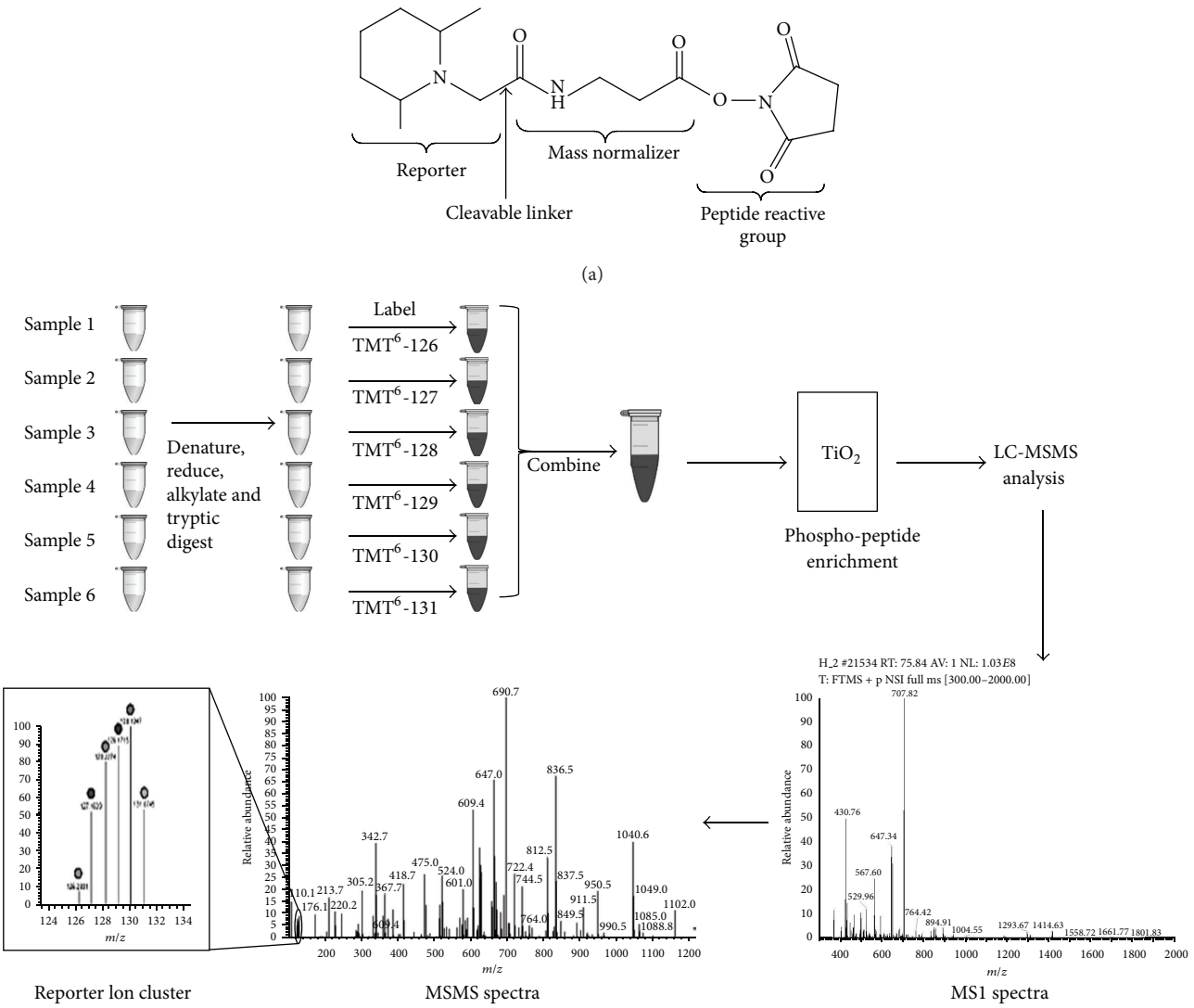

Figure 1: Phospho-TMT workflow. (a) Structure of the TMT Tag. Six tags are available, each with a different reporter group (m/z 126, 127, 128, 129, 130, or 131). The mass normaliser region balances out the difference in mass in the reporter groups such that the overall tag mass is constant. The reactive group provides amine-specific labelling. (b) Six samples are digested with trypsin to generate peptides which are then labelled with one of the six TMT tags. The labelling reaction is quenched and the samples are pooled. The pooled sample is passed through a titanium dioxide (TiO_2) column, phosphopeptides bind to the column while nonphosphorylated peptides pass straight through. The phosphopeptides are then eluted and analysed by LC-MS/MS. Differentially tagged peptides are indistinguishable at the MS1 level since the overall tag mass is constant. Fragmentation of the peptides detected in the MS1 spectra produces secondary MSMS spectra for each peptide, allowing elucidation of the peptide sequence. In addition, the fragmentation process causes cleavage of the linker region within the tag, releasing the reporter groups which appear as a cluster of ions at the low mass end of each MSMS spectra. The relative intensity of the members of this ion cluster shows the relative abundance of that peptide between the six samples under comparison.

ab15000 (Ser16)), myozenin-2 (Santa Cruz sc-377359) at a dilution of 1 : 1000 and with glyceraldehyde 3-phosphate dehydrogenase (Cell signalling 5174) at a dilution of 1 : 10000. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1 : 10000 for 1 h at room temperature. Immunoreactivity was visualized with an ECL reagent (ECL prime western blotting reagent, Amersham, GE Healthcare). They were then exposed to X-ray film. Band intensity was quantified by densitometry using Image J (National Institutes of Health, USA) and the resulting peptides were normalized to GAPDH. Immunoblotting was carried out on samples from the first proteomic run (series 1: 3 control and 3 RIPC).

2.6. Phosphoproteomic Analysis. Analysis of protein phosphorylation in ventricular tissues extracted from both RIPC and control groups was performed using Tandem Mass Tags (TMTs) from Thermo Fisher Scientific (University of Bristol Proteomics Facility, School of Medical Sciences). Details of the methods involved are shown in Figure 1. The analysis was

performed in all twelve hearts used in this study (6 control and 6 RIPC). However, as we could only run 6 samples at a time (3 RIPC and 3 control) the whole processing involved two runs (2 Series). Moreover, the comparison including statistics could only be performed comparing samples (3 control versus 3 RIPC) within one series.

2.6.1. TMT Labelling and Phosphopeptide Enrichment. Aliquots of 100 μg of six samples per experiment were digested with trypsin (2.5 μg trypsin per 100 μg protein; 37°C, overnight) and labelled with Tandem Mass Tag (TMT) 6Plex reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Loughborough, LE11 5RG, UK). Each sample was labelled with a different isobaric tag, RIPC samples were labelled as TMT126, 127, and 128 and for control samples TMT129, 130, and 131. The labelled samples were then combined, evaporated to dryness, resuspended in buffer (57% acetonitrile v/v, 0.4% TFA v/v, 26% lactic acid v/v), and subjected to phosphopeptide enrichment using titanium dioxide TiO_2 Phosphopeptide enrichment kit (Pierce).

2.6.2. Nano-LC Mass Spectrometry. Enriched phosphopeptides were then fractionated using a Dionex Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nanotrap column (Dionex). After washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 250 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Dionex) over a 150 min organic gradient, using 7 gradient segments (1–6% solvent B over 1 min, 6–15% B over 58 min, 15–32% B over 58 min, 32–40% B over 3 min, 40–90% B over 1 min, held at 90% B for 6 min, and then reduced to 1% B over 1 min.) with a flow rate of 300 nl min^{-1}. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nanoelectrospray ionization at 2.0 kV using a stainless steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 250°C. Tandem Mass Spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyse the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 1800 and the top ten multiply charged ions in each duty cycle selected for MS/MS fragmentation using higher-energy collisional dissociation (HCD) with normalized collision energy of 45%, activation time of 0.1 ms and at a resolution of 7500 within the Orbitrap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were used.

The raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific) and searched against the UniProt Human database (122604 entries) using the SEQUEST (Ver. 28 Rev. 13) algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included oxidation of methionine (+15.9949) and phosphorylation at serine, threonine, and tyrosine (+79.966) as variable modifications and carbamidomethylation of cysteine (+57.0214) and the addition of the TMT 6Plex mass tag (+229.163) to peptide N-termini and lysine as fixed modifications. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5%. The Proteome Discoverer software generates a reverse "decoy" database from the same protein database and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identifications. The minimum cross-correlation factor (Xcorr) filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 5% based on the number of random false positive matches from the reverse decoy database. Thus each data set has its own passing parameters. Quantitation was done using a peak integration window tolerance setting of 0.0075 Da with the integration method set as the most confident centroid.

2.7. Statistical Analysis. Statistical analyses were performed using Statveiw for Windows (SAS Institute Inc.). Differences between control and RIPC groups were analyzed using unpaired t-test. Since there were two phosphoproteomic runs with each involving six different samples (3 control and 3 RIPC), the comparison was only made between the two groups for each run.

3. Results

3.1. The Effect of RIPC on Cardiac Phospho-Proteins in Series 1. The first phospho-proteomic analysis run was carried out on 6 samples (3 control and 3 RIPC). Out of a total of ~1700 phosphoproteins measured, only 15 phosphoproteins showed significant increase in RIPC compared to control (Table 1). Most of these proteins are signalling molecules localized to the cardiac sarcomeric Z-disk. Interestingly the RIPC-induced changes in phosphoproteins were similar whether the absolute values were used or when expressed (normalised) per phosphorylated-GAPDH. The latter was detected in all samples.

3.2. Validation of Phosphoprotein Levels in Series 1 Using Western Blotting. Whether the changes in phosphoproteins is a real effect that was validated using western blotting on the same samples that were used for phosphoproteomics. We selected phosphorylated phospholamban to validate phosphoproteomics analysis by western blotting for a number of reasons. First, we detected this protein in the phosphoproteome in all samples. This protein showed significant variation which raised concern as to the phosphoproteomic analysis. Finally, we have the antibody for this protein (phosphorylated at Ser16) which we use routinely in our studies and therefore are very confident using it (Figure 3).

The absolute levels of p-PLB measured for individual samples using TMT isobaric mass tagging were compared to

Table 1: RIPC-induced changes in phosphoproteins measured using TMT. Data shown are for Series 1 (3 control and 3 RIPC). Fold change was calculated by taking average values for control and for RIPC.

Phospho-proteins	Protein accession	Fold change	*P* value	*P* value normalized to p-GAPDH
Myozenin-2	Q9JJW5	1.7	0.021*	0.043*
PDZ and LIM domain protein 5	E9Q8P5	1.3	0.003*	0.057
Protein Pkp2	E0CX59	1.3	0.008*	0.012*
Protein Tns1	E9Q0S6	1.3	0.006*	0.035*
Apoptosis-inducing factor short isoform 2	Q1L6K5	1.2	0.033*	0.040*
Isoform 2 of AP2-associated protein kinase 1	Q3UHJ0-2	1.4	0.014*	0.013*
Isoform 3 of synaptopodin	Q8CC35-3	1.3	0.036*	0.094
Ryanodine receptor 2	E9Q401	1.2	0.043*	0.196
Obscurin	F7DCJ0	1.2	0.012*	0.32
Alpha-T-catenin isoform X	A4GE65	1.2	0.033*	0.159
Ubiquitin carboxyl-terminal hydrolase	B1AY13	1.2	0.029*	0.031*
Sorbin and SH3 domain-containing protein 2	B2RXQ9	1.4	0.007*	0.050
ENH isoform 1e	D9J302	1.2	0.003**	0.051
p53-induced protein with a death domain	Q9ERV7	1.3	0.034*	0.092
Calnexin	P35564	1.2	0.071	0.013*

P-value calculated using unpaired *t*-test (*$P < 0.05$ considered as significant). *P* values between 0.05–0.1 indicate strong trend.

Table 2: RIPC-induced changes in phosphoproteins measured using TMT. Data shown are for the second group of hearts (Series 2; 3 control and 3 RIPC). Fold change was calculated by taking average values for control and for RIPC.

Phosphoproteins	Protein accession	Fold change	*P* value	*P* value normalised to p-GAPDH
Myozenin-2	Q9JJW5	1.9	0.015*	0.020*
Tropomyosin alpha-1 chain	P09493	1.5	0.036*	0.08
Troponin I	P48787	1.6	0.029*	0.043*
M-protein	O55124	1.4	0.023*	0.016*
Eif4g1 protein	Q8R2V4	1.3	0.010*	0.040*
Isoform 2 of titin	A2ASS6-2	1.6	0.061	0.12
Junctophilin-2	Q9BR39	1.7	0.070	0.053
Isoform A3B of troponin T, cardiac muscle	P50752-2	1.2	0.06	0.064
Ataxin-2	F6V8M6	0.8	0.005*	0.043*
Isoform Tau-C of microtubule-associated protein tau	P10637-4	0.8	0.008*	0.050
Centrosomal protein of 170 kDa	H7BX26	0.8	0.031*	0.072
Protein kinase, cAMP dependent regulatory, type I, alpha	A2AI69	0.7	0.019*	0.031*
THUMP domain-containing protein 1	Q99J36	0.8	0.028*	0.09
CDKN2A-interacting protein	Q9NXV6	0.8	0.060	0.103

P-value calculated using unpaired *t*-test (*$P < 0.05$ considered as significant). *P* values between 0.05–0.1 indicate strong trend.

levels measured using Western blot for p-PLB and corrected for GAPDH. As shown in Figure 2 the levels of p-PLB in different samples followed a similar pattern whether using phosphoproteomics or Western blotting.

3.3. The Effect of RIPC on Cardiac Phosphoproteins in Series 2. The second phosphoproteomic analysis run was carried out on an extra, different 6 samples (3 control and 3 RIPC). The analysis of these new samples detected ~2300 phosphoproteins. However, only 14 phosphoproteins showed significant differences between RIPC and control (Table 2). Most of these proteins are also signalling molecules localized to the cardiac sarcomeric Z-disk. The protein that showed the highest change in the level of phosphorylation in both series was myozenin-2 (also known as Calsarcin 1). This is a novel family of sarcomeric calcineurin-binding proteins.

3.4. The Effect of RIPC on Myozenin-2 (Calsarcin1) Phosphorylation. RIPC significantly increased myozenin-2 phosphorylation compared to the control group in both runs (Tables 1 and 2). The following phosphorylation sites were detected; threonine 107, serine 106, and serine 101 (Table 3). Unlike phosphorylated myozenin-2, the total myozenin-2 measured using western blotting did not show significant changes between RIPC and control (Figure 4). There are

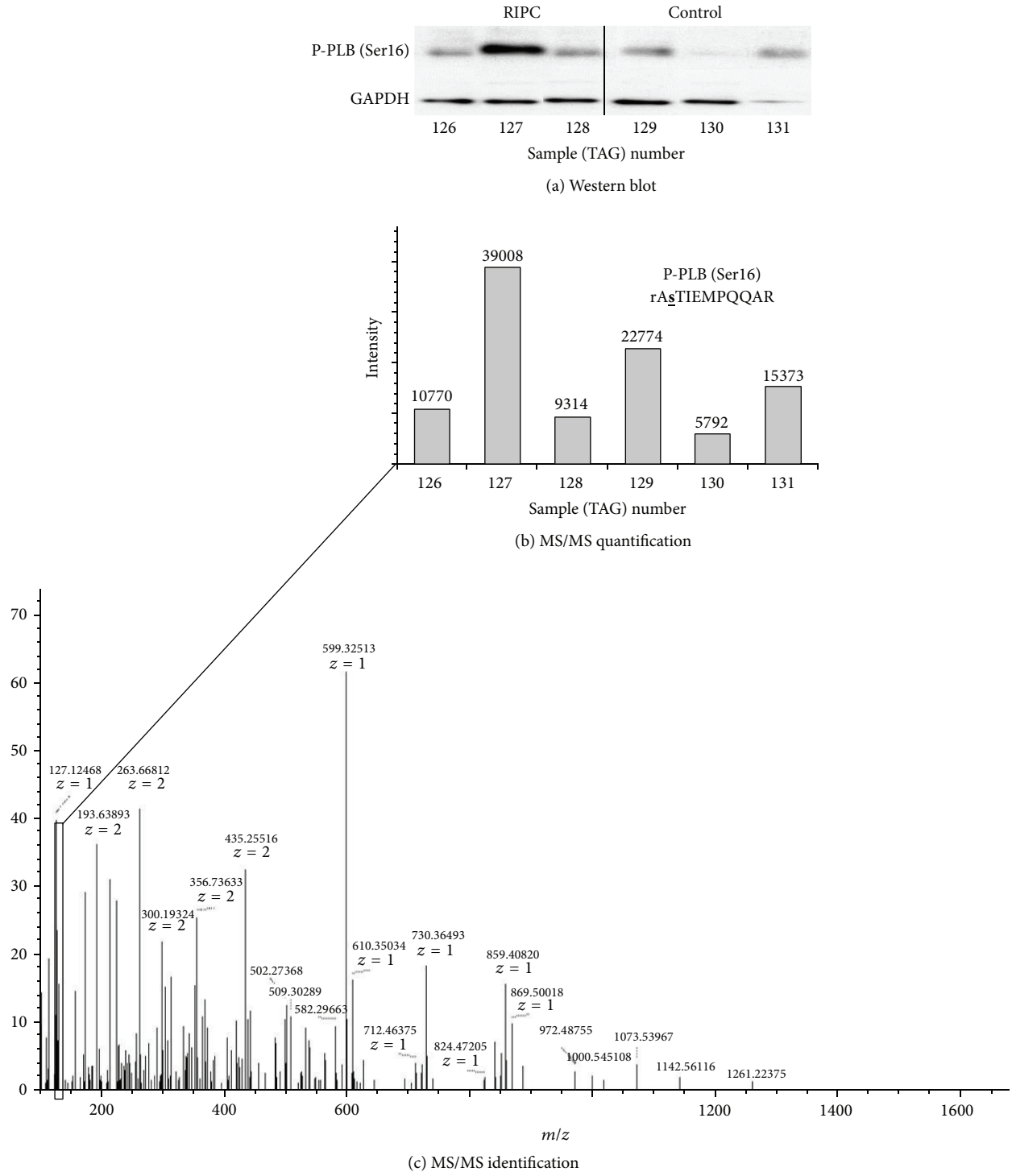

Figure 2: Quantification of phosphorylated phospholamban (p-PLB) in the same samples measured using Western blotting or TMT tandem mass tagging. Protein expression level for cardiac p-PLB (GAPDH in lower panel) in RIPC (126, 127, and 128) and control (129, 130, and 131) samples are shown in (a). Raw data for identification (c) and quantification (b) of the phosphopeptide RASTIEMPQQAR from PLB using TMT isobaric mass tagging are shown in (b) & (c). The reporter ion cluster from the six different mass tags are shown boxed in panel (c) and expanded in bar chart form in panel (b) for both RIPC (numbers: 126, 127, and 128) and control (129, 130, and 131) samples. The peptide sequence showing the phosphorylated amino acid (Ser 16, underlined) is also shown in panel (b). The samples used in these measurements were taken from series 1.

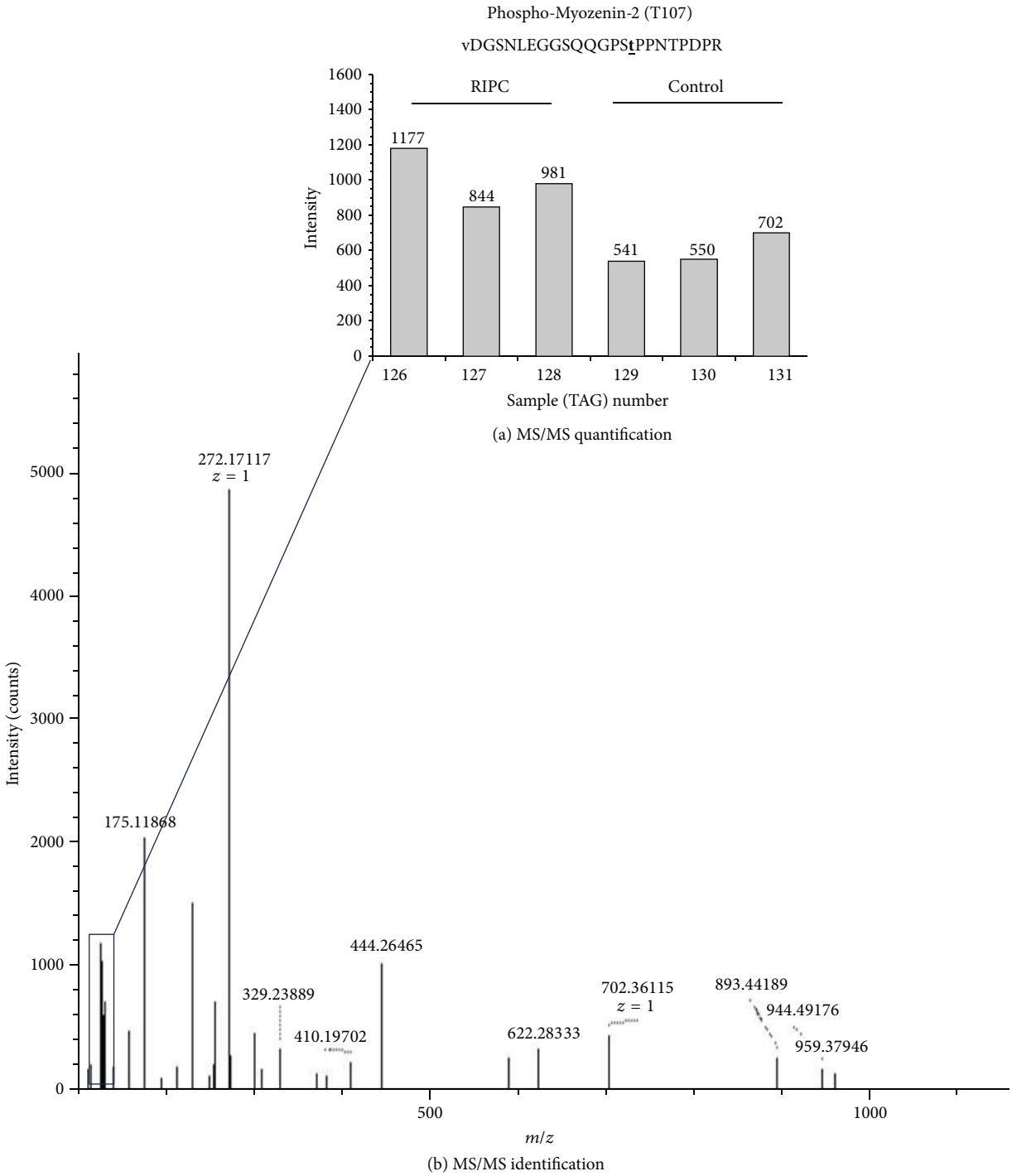

Figure 3: Phosphopeptide identification and quantification for phospho-myozenin-2 from RIPC and control samples using Tandem Mass Tagging. P-myozenin-2 peptide phosphorylated at threonine 107 is shown in panel (a). The mass peaks generated by different mass tags (reporter ions) for different samples are magnified and shown in bar chart form in panel (a) for both RIPC (numbers: 126, 127, and 128) and control (129, 130, and 131) samples. The (MS/MS) spectra for the peptide used for protein identification and the reporter ions (boxed) are shown in (b). The samples used in these measurements were taken from Series 1.

Table 3: RIPC-induced changes in phosphorylation of myozenin-2 (protein accession: Q9JJW5) as per phosphorylation sites (amino acids shown in small bold and underlined letters). Phosphorylated myozenin-2 was measured using TMT isobaric mass tagging. Data shown are for series 1 (3 control and 3 RIPC). Fold change was calculated by taking average values for control and for RIPC.

Amino acid sequence of fragments	Modified residue(s)	Fold change	*P* value	*P* value normalised to p-GAPDH
vDGSNLEGGSQQGPS**t**PPNTPDPR	T107	1.67	0.021*	0.043*
vDGSNLEGGSQQGP**s**TPPNTPDPR	S106	1.55	0.007*	0.025*
vDGSNLEGG**s**QQGPSTPPNTPDPR	S101	1.34	0.017*	0.078
vDGSNLEGG**s**QQGP**s**TPPNTPDPR	S101/S106	1.15	0.290	0.169
vDGSNLEGG**s**QQGPS**t**PPNTPDPR	S101/T107	1.09	0.665	0.637
vDGSNLEGGSQQGP**s**TPPN**t**PDPR	S106/T111	1.15	0.290	0.169
vDGSNLEGGSQQGPS**t**PPN**t**PDPR	T107/T111	1.09	0.665	0.637

P value calculated using unpaired *t*-test (* $P < 0.05$ considered as significant).

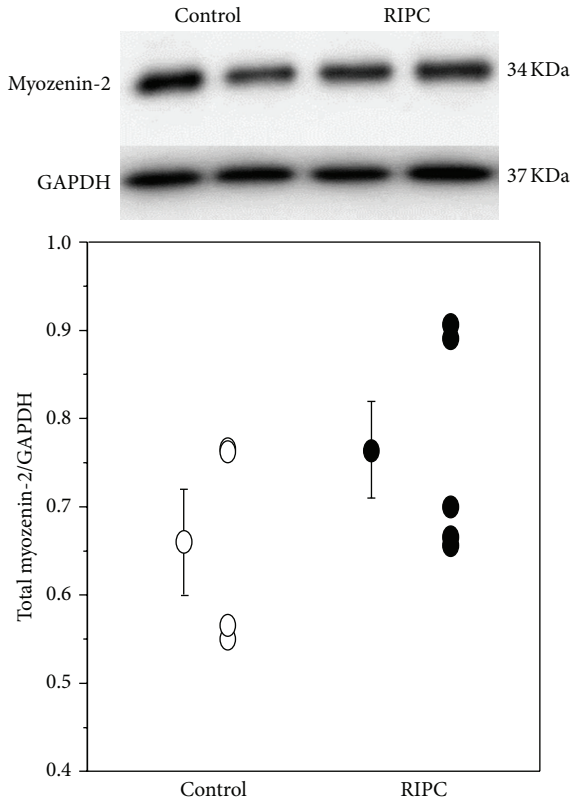

Figure 4: Total myozenin-2 in ventricular tissues extracted from RIPC ($n = 5$) and control ($n = 4$) samples. Upper panel is a representative blot showing myozenin-2 and GAPDH. The graph shows individual myozenin-2 levels (normalized to GAPDH) as well as the mean ± SEM for each intervention. There was no statistical difference between the two groups.

no antibodies available for phosphorylated myozenin-2 and therefore did not perform western blot for this protein.

4. Discussion

The mechanism by which RIPC protects the myocardium against ischemia/reperfusion (I/R) injury is not clear; however, some proposed pathways suggest the release of substances from the remote organ which activate a complex intracellular signaling cascade in myocardium. It is proposed that these signalling pathways act either via the activation of the potassium-dependent ATP (K_{ATP}) channel [34] and/or by inhibiting the opening of the mitochondrial permeability transient pore to induce cardioprotection [35]. The involvement of these pathways has been demonstrated during reperfusion with little work describing protein activation at the end of RIPC and before I/R. Therefore we performed a phosphoproteomic study to identify proteins that are activated immediately after RIPC.

Cardiac proteomic studies provide insight into changes in total protein expression during cardiac disease and for the identification and localization of posttranslational modifications with the hope that this technique will also help to identify markers of cardiac disease as well as novel therapeutic targets [36–41]. Two-dimensional gel electrophoresis has been used to visualize the proteome profile in the border zone of the early stage postinfarct [42] and proteins that were differentially expressed in the ischemically reperfused heart compared with the control and ischemically preconditioned rat hearts [43]. Proteomic blood study during RIPC study using 2D-gel suggested either neurogenic pathway or humoral factor less than 8 kDa as possible mediators [44].

A recent study by Hepponstall et al. (2012) examined the global proteomic changes that occur during RIPC in plasma. They identified six proteins that changed in response to RIPC using 2D DIGE analysis, while 48 proteins were found to be differentially regulated using LC-MS [45]. These changes were cumulative with each episode of RIPC and the proteins identified have a range of cellular functions including immune response, homeostasis, cell adhesion, and lipid transport. Recent years have witnessed the introduction of gel-free proteomic approaches. These include isotope-coded affinity tagging (ICAT), SILAC, and isobaric tag for relative and absolute quantification (iTRAQ) [46]. These LCMS-based approaches provide considerably more information than the more classic 2D gel-based approaches.

In our study we used Tandem Mass Tagging, (TMT) combined with phosphopeptide enrichment to determine the changes in the cardiac phosphoproteome during the cardioprotective intervention, RIPC. The technique was also confirmed and validated using western blotting. Several of the phosphoproteins identified were associated with Z-disk sarcomere.

Our phosphoproteomic analysis showed that RIPC is associated with an increase of phosphorylation of several proteins most of which are found in the Z-disk of the sarcomere including: myozenin-2, Obscurin, PDZ, and LIM domain protein 5, myopalladin and several other Z-disk proteins. In addition to their structural function, these proteins are known to play a role in signal transduction [47]. A number of signaling proteins including protein kinases and phosphatases are concentrated at the Z-disk where they interact with other Z-disk proteins which function as sensors of mechanical stress [47, 48]. In particular, PKCε is localized to the Z-disk [49] and it translocate to the nucleus upon biomechanical stress [50, 51]. PKCε has also been implicated in the cardioprotective effect of RIPC [29] and its activation is cardioprotective [52]. PDZ and LIM domain protein 5 (Table 1) has been shown to interact with PKC by scaffolding PKC to the Z-disk region [53]. Plakophilin 2 (PKP2) (Table 1) also serves as scaffold for PKCα signalling [54, 55].

Obscurin is a modular protein of ~800 kD which contains a GTPase nucleotide exchange factor (GEF) domain that provides a possible link between the sarcomere and the G-protein regulated pathways which control the formation of new myofibrils [56]. Obscurin also binds to ankyrin 1, which links the sarcoplasmic reticulum to the sarcomere and appears to be involved in the regulation of ryanodine receptor distribution [57]. Myozenin-2 is a calcineurin-binding protein and plays an important role in hypertrophic cardiomyopathy through its effect on calcineurin activity [58]. It has also been shown to protect against angiotensin-II induced cardiac hypertrophy [59].

What remains unclear is how myozenin-2 is regulated posttranscriptionally. A large scale phosphorylation analysis reported phosphorylation of myozenin-2 on T-107 and T-111 from mouse liver [60]. These modifications were also detected in the heart along with two other phosphorylation sites on S106 and S116 [61, 62]. In this study we detected increased level of phosphorylation of myozenin-2 on T-107, S-106, and S-101 immediately following RIPC compared to control (Table 3). Phosphorylation of myozenin-1 and myozenin-2 has been proposed to occur by protein kinase A (PKA) at residues within PDZ-binding motif [63]. It is known that PKA phosphorylates several sarcomeric proteins during stimulation of α-adrenergic receptors on myocytes [64]. Additionally activation of PKA could be responsible for the increased phosphorylation of ryanodine receptor 2 (Table 1) and troponin I (TnI) (Table 2). Phosphorylation of TnI by PKA is seen upon stimulation of the heart by β-agonists which results in a decrease in the Ca^{2+} sensitivity of muscle contraction [65]. It is worth noting that in one of the runs (series 2) we found decreased phosphorylation in cAMP dependent regulatory, type I, alpha unit of protein kinase (Table 2). Whether this would alter the activity of PKA is not presently known.

In conclusion, this study reports the novel finding that RIPC triggers changes in phosphorylation levels of cardiac proteins including several located to the Z-disk area of the sarcomere. Of particular interest is p-myozenin-2 which we found to be significantly higher in RIPC compared to control hearts. The increased phosphorylation was seen at different phosphorylation sites. Z-disk proteins are involved in signalling pathways and may protect the heart against R/I. Additionally; several kinases involved in survival signalling (e.g., PKA, PKC, and PKG) are also linked to the phosphorylation of these proteins. Further studies are needed to identify their role in cardioprotection during RIPC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Age and SPARC Change the Extracellular Matrix Composition of the Left Ventricle

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Secreted protein acidic and rich in cysteine (SPARC), a collagen-binding matricellular protein, has been implicated in procollagen processing and deposition. The aim of this study was to investigate age- and SPARC-dependent changes in protein composition of the cardiac extracellular matrix (ECM). We studied 6 groups of mice (n = 4/group): young (4-5 months old), middle-aged (11-12 m.o.), and old (18–29 m.o.) C57BL/6J wild type (WT) and SPARC null. The left ventricle (LV) was decellularized to enrich for ECM proteins. Protein extracts were separated by SDS-PAGE, digested in-gel, and analyzed by HPLC-ESI-MS/MS. Relative quantification was performed by spectral counting, and changes in specific proteins were validated by immunoblotting. We identified 321 proteins, of which 44 proteins were extracellular proteins. Of these proteins, collagen III levels were lower in the old null mice compared to WT, suggestive of a role for SPARC in collagen deposition. Additionally, fibrillin showed a significant increase in the null middle-aged group, suggestive of increased microfibril deposition in the absence of SPARC. Collagen VI increased with age in both genotypes (>3-fold), while collagen IV showed increased age-associated levels only in the WT animals (4-fold, $P < 0.05$). These changes may explain the previously reported age-associated increases in LV stiffness. In summary, our data suggest SPARC is a possible therapeutic target for aging induced LV dysfunction.

1. Introduction

Age is a prominent risk factor for increased cardiovascular morbidity and mortality, and the incidence of cardiovascular diseases such as hypertension and myocardial infarction (MI) are higher in individuals over 65 years of age [1]. Aging has been associated with significant structural changes in the left ventricle (LV) and data from several clinical trials show evidence that elderly patients have poorer outcomes after ischemic stress [2, 3]. The age-related decline in function of the cardiovascular system is associated with myocyte loss and a subsequent increase in the cardiac extracellular matrix (ECM) [4–6]. Evolving evidence suggests that cardiac senescence by itself affects myocardial structure and function that can affect how one responds to additional cardiac stressors. However, the frequent presence of comorbidities has hindered the identification of cardiac age-related therapeutic targets.

Myocardial ECM is composed of collagens, proteoglycans, glycoproteins, extracellular proteases, and ECM receptors [7, 8]. Among cardiac ECM components, fibrillar collagens provide myocardial structural support, mechanical stability, and morphology [9]. The collagenous matrix supports and aligns cardiomyocytes and vasculature and coordinates cell migration and proliferation [9, 10]. Experimental models have shown that excessive accumulation of fibrillar collagens in aged animals can lead to a significant decline in diastolic function [11, 12]. The major fibrillar collagens present in the myocardial ECM are collagen type I (approximately 85%, percentage is species dependent) and collagen type III (approximately 11%) [13]. These collagens display high tensile strength, which plays an important role in LV function [13]. Several experimental studies have clearly demonstrated a direct relation between increased collagen content and increased myocardial stiffness [14–16]. The factors responsible for increased collagen content with age are not well understood. However, cardiac aging is characterized by a loss of cardiomyocytes, and this may explain the increased collagen deposition in the LV walls [17]. Other factors that may be involved in age-associated fibrosis are inhibition of collagen degradation by changes in cardiac matrix metalloproteinases and respective tissue inhibitors of metalloproteinases and increased collagen fibril crosslinking and assembly [18].

Secreted protein acidic and rich in cysteine (SPARC), a matricellular protein, is known to regulate collagen fibril morphology and assembly [19]. SPARC has also been reported to regulate various cellular processes, including cell migration, proliferation, tissue morphogenesis, and tissue repair [20, 21]. In a mouse model, aging was associated with increased expression of SPARC and increased insoluble and fibrillar collagen content, which related to increased cardiac stiffness [2]. SPARC deletion blunted the aging-related effects. To further study the impact of this matricellular protein in cardiac ECM and aging, we developed an enrichment protocol coupled with a proteomic approach to analyze SPARC and age-dependent changes in the expression and accumulation of cardiac ECM proteins. This proteomics study used a mass spectrometry (MS) approach to identify low abundant ECM proteins in the cardiac matrix and immunoblotting to quantify protein changes that occur with age that are SPARC dependent. We tested the hypotheses that cardiac ECM protein content is influenced by progressive aging and that SPARC plays a key role in the structure and composition of the aged myocardium.

2. Materials and Methods

2.1. Animals and LV Collection. Mice colonies were maintained at the Medical University of South Carolina (MUSC) animal care facility. All procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, The National Academies Press, Washington, DC, 2011) and were approved by the Institutional Animal Care and Use Committee at MUSC (Approval ID: ACORP 511). We used C57BL/6 wild type (WT) and SPARC-null mice to study age, sex, and genotype differences in the left ventricle (LV). Three age groups were studied: young (4-5 month old), middle-aged (11-12 month old), and old (18–29 month old), and both male and female mice were included in each group (n = 4/sex/age/genotype). The generation and phenotype of SPARC-null mice have been previously described by Norose and colleagues [22]. Animals were anesthetized with isoflurane and hearts excised. The hearts were washed in phosphate saline buffer (PBS) and the LV separated from the right ventricle. The LV was used for all further studies.

2.2. Tissue Decellularization. Whole LVs were decellularized as previously reported [23]. In summary, the tissue was incubated in decellularization buffer (1% sodium dodecyl sulfate in PBS) with 1x protease inhibitors cocktail (PI; cOmplete Mini tablets, Roche). Samples were left at room temperature in an orbital shaker until tissue was completely decellularized (three to four days). The decellularization buffer was decanted daily and replaced with fresh decellularization buffer. When tissue looked translucent, samples were considered decellularized. Tissue was washed three times in distilled water with 1x PI for 5 min and then left in fresh 1x PI/water overnight to remove all remnants of the decellularization buffer. The decellularized LVs were homogenized (Power Gen 1000, Fisher Scientific) in Protein Extraction Reagent Type 4 (7.0 M urea, 2.0 M thiourea, 40 mM Trizma base, and 1.0% C7BzO, pH 10.4) and 1x PI. Protein quantification was performed using a Coomassie Brilliant Blue G-250-based assay (Quick Start Bradford Protein Assay, Bio-Rad). All samples were stored at −80°C until use.

2.3. Mass Spectrometry. Proteins (10 μg, n = 4 per age group/genotype) were separated by 1D SDS-PAGE in a 4–12% Bis-Tris gel and stained with EZBlue (Sigma Aldrich), which is a Coomassie Brilliant Blue-based dye compatible with MS analysis. The gel lane for each sample was divided into three slices, which contained the visually detectable proteins. Each slice was individually destained and dehydrated, and the proteins digested *in situ* with trypsin (Promega). The digests were analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) on a Thermo Fisher LTQ Orbitrap Velos mass spectrometer fitted with a New Objective Digital PicoView 550 NanoESI source. Online HPLC separation of the digests was accomplished with an Eksigent/AB Sciex NanoLC-Ultra 2D HPLC system: column, PicoFrit (New Objective; 75 μm i.d.) packed to 15 cm with C18 adsorbent (Vydac; 218 MS 5 μm, 300 Å). Precursor ions were acquired in the Orbitrap in profile mode at 60,000 resolution (m/z 400); data-dependent collision-induced dissociation (CID) spectra of the six most intense ions in the precursor scan above a set threshold were acquired sequentially in the linear trap at the same time as the precursor ion scan. Mascot (version 2.3.02; Matrix Science) was used to search the mass spectra against a combination of the mouse subset of the NCBInr database (Mus. (145,083 sequences)) and a database of common contaminants (179 sequences). Methionine oxidation was considered as a variable modification; trypsin was specified as the proteolytic enzyme, with one missed cleavage

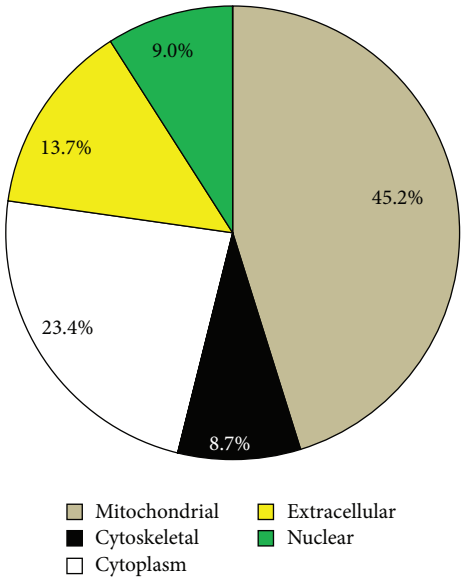

Figure 1: Decellularization of the left ventricles enriched for extracellular proteins, including secreted and membrane proteins. Using mass spectrometry, we identified a total of 321 proteins, of which 44 (13.7%) were extracellular proteins.

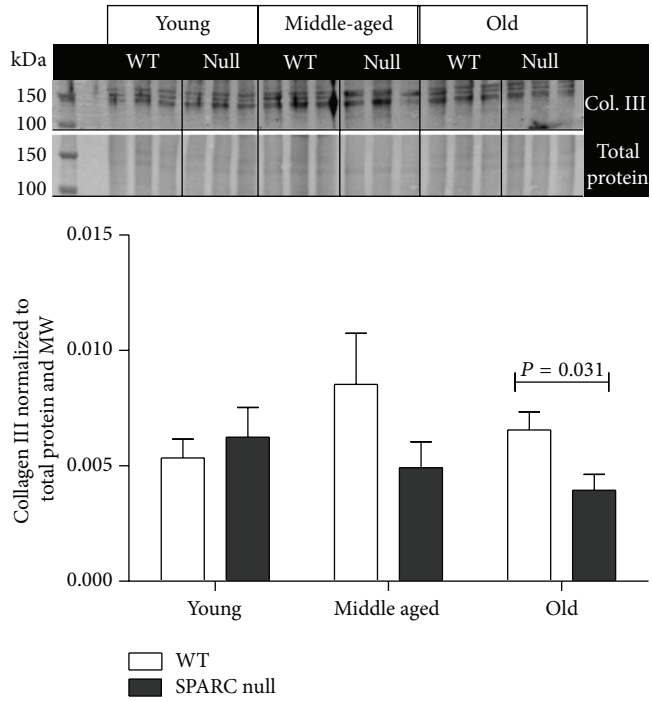

Figure 2: In the old mice, LV collagen III levels were lower in the SPARC null mice compared to the WT. Protein levels were quantified by immunoblot (n = 6/genotype/age). Two controls were used, total protein stain (loading control) and molecular weight marker (MW, blot to blot control). The signal intensity of the MW was used to normalize the data among blots, while protein levels were normalized to the total protein in its respective lane. Values were plotted as mean ± SEM.

allowed. The Mascot data files were combined in Scaffold (Proteome Software; version 3) for a subset search of the mass spectra using X! Tandem, cross correlation of the X! Tandem and Mascot results, and determination of probabilities of peptide assignments and protein inferences. The thresholds for acceptance of peptide and protein assignments in Scaffold were 95% and 99%, respectively, and minimum of one unique peptide.

2.4. Immunoblots. An aliquot of each sample (10 μg protein) was loaded onto a 4–12% Bis-Tris gel and separated by 1D SDS-PAGE electrophoresis. Proteins were transferred to a nitrocellulose membrane, which was treated with the MemCode Reversible Protein Stain Kit (Pierce, Thermo Scientific) to check for efficiency of protein transfer and for use as a loading control. Destained membranes were blocked for 1 h at room temperature with 5% nonfat milk (Bio-Rad) and hybridized overnight at 4°C with primary antibodies against the following: collagen types I, III, IV, and VI (Cedarlane CL50141AP-1, CL50341AP-1, CL50441AP-1, and Abcam ab6588), fibrillin (Cosmobio LSL-LB-2297), and laminin beta 2 (Novus Biologicals NBP1-00904). After 1 h incubation with a secondary antibody, positive signaling was detected by chemiluminescent using an ECL substrate (GE Healthcare). Immunoblots were densitometrically analyzed using GE Image Quant LAS4000 luminescent image analyzer (GE Healthcare). The signal intensity of each sample was normalized to the total protein in its respective lane. Data are reported as mean ± SEM. The Kruskall-Wallis non-parametric test was used to assess differences among groups, and the Dunn's multiple comparison post-test was used when differences were observed. A $P < 0.05$ was considered significant.

3. Results and Discussion

We identified 321 proteins by mass spectrometry, of which 44 proteins (13.7%) were extracellular, including secreted and cell membrane proteins (Figure 1). We used normalized spectrum counts to perform relative quantification and identify age- and SPARC-related changes (Table 1). In accordance with previous reports, the fibrillar collagens alpha 1(I) and alpha 2(I) showed an age-dependent increase that was blunted with SPARC deletion [9, 18, 24]. With age, the levels of total collagen and insoluble collagen, collagen fibril diameter, and the extent of collagen cross-linking all increase [9, 18, 24]. These changes lead to increased LV stiffness and cardiac dysfunction [25, 26]. Upon secretion, collagen molecules are processed and stabilized by the formation of covalent cross-links resulting in mature cross-linked collagen, processes influenced by SPARC [27]. The relationship between SPARC and postsynthetic procollagen processing suggests that SPARC deletion may diminish the decline in diastolic function observed with aging.

We found that collagen III levels were reduced in the old null mice compared to WT (Figure 2). In a normal young adult heart, collagen III constitutes approximately 11% of the total cardiac collagen content [13]. Several studies have shown that during the aging process cardiac collagen III content

Table 1: Quantitative values (normalized weighted spectra) for the identified extracellular proteins ($n = 4$ per group, values are mean ± SEM).

Identified Proteins	Molecular weight	Young WT	Young KO	Middle WT	Middle KO	Old WT	Old KO	Accession number
Secreted proteins								
Annexin A2	39 kDa	0	0	0	0	0.5 ± 0.5	0.5 ± 0.5	gi|12849385
Apolipoprotein O	21 kDa	0	0.8 ± 1.0	0	0	0	0	gi|123122452
Collagen type I alpha-1	138 kDa	10.0 ± 0.4	7.8 ± 2.6	12.5 ± 2.6	7.0 ± 0.8	10.5 ± 1.2	6.0 ± 1.8	gi|34328108
Collagen type I alpha-2	130 kDa	6.0 ± 0.7	3.5 ± 1.3	9.5 ± 1.6	3.5 ± 1.3	9.3 ± 1.4	7.0 ± 0.6	gi|111120329
Collagen type III alpha-1	139 kDa	0.3 ± 0	0.3 ± 0	1.5 ± 0.8	0.5 ± 0	1.3 ± 0	0.8 ± 0	gi|74184771
Collagen type IV alpha-2	167 kDa	1.5 ± 0.6	0.8 ± 0.6	3.8 ± 0.6	2.3 ± 0.6	4.3 ± 0.8	3.0 ± 0	gi|556299
Collagen type VI alpha-1	108 kDa	7.8 ± 0.6	8.8 ± 0.5	7.5 ± 1.2	7.8 ± 1.9	6.0 ± 1.1	6.3 ± 0.6	gi|6753484
Collagen type VI alpha-2	110 kDa	4.0 ± 0.9	6.0 ± 1.2	7.8 ± 1.5	9.5 ± 1.4	11.0 ± 0.7	9.5 ± 1.7	gi|22203747
Collagen type VI alpha-3	287 kDa	41.8 ± 6	46.3 ± 7	45.0 ± 7	51.5 ± 7	51.3 ± 5	48.0 ± 4	gi|148708135
Collagen type XV	140 kDa	0	0	0	0.3 ± 0.3	0	0	gi|11037306
Collagen type XXIV alpha-1	176 kDa	0	0	0.3 ± 0.3	0	0	0	gi|116326001
Fibrillin-1	312 kDa	0	0.3 ± 0.3	0	0	0	0.5 ± 0.5	gi|118197277
Fibrinogen, alpha	87 kDa	0.3 ± 0	0	0	1.0 ± 0.6	3.3 ± 1.7	3.0 ± 1.3	gi|148683476
Fibrinogen, beta	55 kDa	0.0	0	0.3 ± 0	0.5 ± 0.5	1.3 ± 0.9	0.5 ± 0.5	gi|33859809
Fibrinogen, gamma	49 kDa	0.0	0	0	0	0.5 ± 0.5	0	gi|148683478
Laminin alpha-2	344 kDa	0.8 ± 0	1.5 ± 0.7	0.5 ± 0.5	0.3 ± 0.3	0	0.3 ± 0.3	gi|117647249
Laminin beta-1	204 kDa	1.0 ± 0.7	0.5 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	1.0 ± 0.6	0.3 ± 0.3	gi|148704971
Laminin beta-2	197 kDa	0.3 ± 0.3	1.5 ± 0.9	0.5 ± 0.5	1.5 ± 0.6	0.5 ± 0.3	1.3 ± 0.5	gi|31982223
Laminin gamma-1	179 kDa	1.8 ± 0.5	1.0 ± 0.4	2.0 ± 0.8	5.5 ± 1.2	2.3 ± 0.9	2.5 ± 0.6	gi|148707495
Nidogen-1	137 kDa	1.0 ± 0.6	1.3 ± 0.5	0	0.3 ± 0.3	0.5 ± 0.5	0.8 ± 0.3	gi|171543883
Perlecan	470 kDa	4.8 ± 0.5	4.8 ± 0.3	3.5 ± 1.0	5.5 ± 1.2	5.8 ± 0.8	6.8 ± 1.3	gi|183979966
Prelamin-A/C	74 kDa	0.3 ± 0	0.8 ± 0.5	0.3 ± 0	0	0	0	gi|162287370
Serum albumin	69 kDa	0	0	0	1.0 ± 0.5	0.3 ± 0.3	0	gi|163310765
Troponin I, cardiac muscle	24 kDa	15.8 ± 0.9	13.5 ± 1.2	11.8 ± 2.1	14.3 ± 0.9	10.8 ± 2.6	9.8 ± 2.0	gi|6678393
Troponin T2, cardiac	32 kDa	0.8 ± 0.5	1.0 ± 0.7	0.5 ± 0.5	0	0	0	gi|148707615
Von Willebrand factor A domain-containing protein 8	213 kDa	2.3 ± 0.6	3.5 ± 0.6	1.8 ± 0.4	2.0 ± 0.5	3.0 ± 0.5	3.0 ± 0.8	gi|226958579
Cell membrane proteins								
Alpha-sarcoglycan	43 kDa	0	0	0	0	0	0.3 ± 0.3	gi|2411510
Aminopeptidase	103 kDa	0	0.5 ± 0.5	0.5 ± 0.5	1.5 ± 1.0	0	0.5 ± 0.5	gi|1184161
ATP1a1 protein	108 kDa	5.3 ± 1.9	6.8 ± 2.9	4.3 ± 3.0	6.5 ± 3.8	2.0 ± 0.8	1.3 ± 0.8	gi|16307541
ATP-binding cassette (ALD) 3	75 kDa	0	0	0	0	0	0	gi|14318642
ATP-binding cassette (MDR/TAP) 8	78 kDa	0.8 ± 0.5	0.0	0.5 ± 0.5	0	0	0	gi|148671187
EH domain-containing protein 4	61 kDa	2.0 ± 0.7	1.5 ± 0.9	2.8 ± 0.9	4.0 ± 0.4	1.5 ± 0.5	2.8 ± 0.3	gi|31981592
Guanine nucleotide-binding protein, subunit beta-2	37 kDa	0	1.3 ± 0.6	0.5 ± 0.2	0.5 ± 0.3	0.5 ± 0.3	0	gi|13937391
Guanosine diphosphate dissociation inhibitor 2	53 kDa	0	0	0	1.0 ± 1	0	0	gi|148700276
Neutral cholesterol ester hydrolase 1	46 kDa	4.5 ± 0.3	3.0 ± 0.4	0	0.3 ± 0.3	0	0.5 ± 0.5	gi|30520239
PDZ and LIM domain protein 5	24 kDa	2.5 ± 0.5	3.3 ± 0.6	1.5 ± 0.6	2.0 ± 0.4	0.5 ± 0.3	0.8 ± 0.8	gi|300069034
Perilipin-4	139 kDa	3.5 ± 1.2	5.0 ± 0.8	4.8 ± 0.6	6.0 ± 2.0	8.0 ± 2.0	6.8 ± 1.6	gi|157041252
Peroxisomal membrane protein 20	17 kDa	2.3 ± 0.3	3.5 ± 0.5	1.3 ± 1.3	1.5 ± 0.3	1.5 ± 0.5	1.5 ± 0.9	gi|6746357
Platelet glycoprotein 4	53 kDa	0	1.3 ± 1.3	0.5 ± 0.5	0	0	0	gi|74151899
Protease, serine 15	109 kDa	0.5 ± 0.5	0	0	0	0	0	gi|148706233
Sodium/calcium exchanger 1	108 kDa	0.5 ± 0.5	0.8 ± 0.8	0	0	0	0	gi|119120890

Table 1: Continued.

Identified Proteins	Molecular weight	Young WT	Young KO	Middle WT	Middle KO	Old WT	Old KO	Accession number
Sorbin and SH3 domain-containing protein 1	83 kDa	3.5 ± 1.6	4.0 ± 0.9	6.3 ± 0.6	5.0 ± 0.9	6.5 ± 0.9	5.3 ± 1.3	gi|78000154
Transglutaminase	77 kDa	0	0	0	0	0	0.3 ± 0.3	gi|201941
Tripartite motif-containing protein 72	53 kDa	6.5 ± 1.3	8.0 ± 0.8	10.0 ± 0.7	10.3 ± 1.8	7.8 ± 1.3	11.3 ± 1.1	gi|121247302

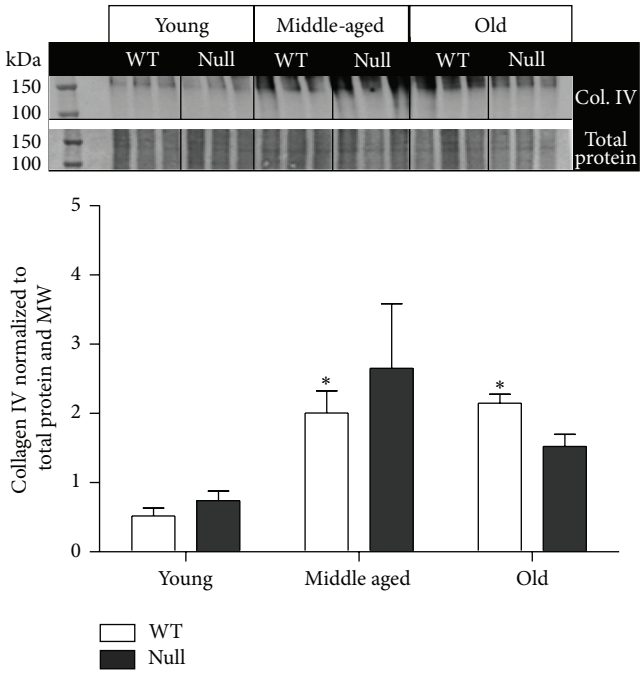

Figure 3: Collagen IV expression levels increased with age in the wild type (WT), but not in the SPARC-null (null) mice. Collagen IV protein levels were quantified by immunoblot (n = 6/genotype/age). Two controls were used, total protein stain (loading control) and molecular weight marker (MW, blot to blot control). The signal intensity of the MW was used to normalize the data among blots, while protein levels were normalized to the total protein in its respective lane. Values were plotted as mean ± SEM; $^{*}P < 0.05$ versus respective young group.

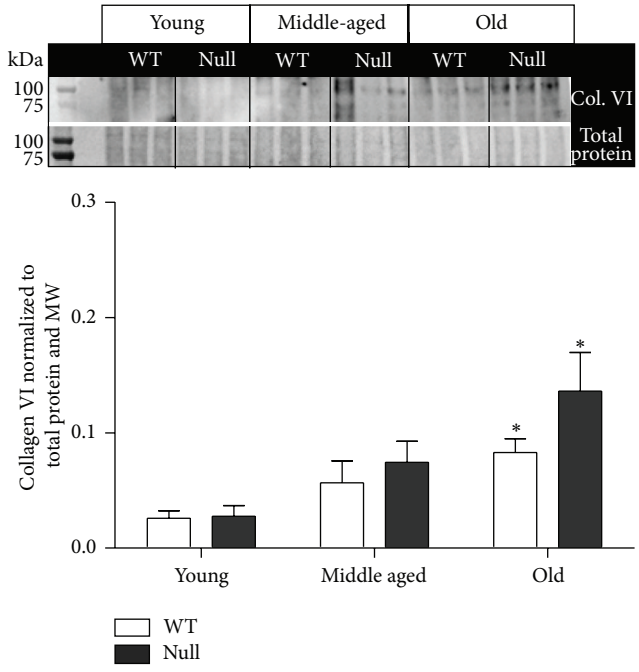

Figure 4: LV levels of collagen VI gradually increased with age. This increase was not SPARC dependent. Values were plotted as mean ± SEM; n = 6/genotype/age; $^{*}P < 0.05$ versus respective young group.

gradually increases but at a lower rate when compared to collagen I [28, 29]. SPARC deletion or inhibition in the elderly may be a target of interest for the treatment of age-related cardiac fibrosis.

Interestingly, the nonfibrillar collagen type IV increased with age in the WT mice but not in the SPARC null group (Figure 3). Collagen type IV is a reticular basement membrane type collagen that plays a fundamental role during embryonic cellular differentiation, proliferation, survival, and migration [30]. Collagen IV can form complex structural scaffolds, which are covalently linked and are required for basement membrane assembly [31, 32]. To date, there are no studies on collagen type IV in the aged heart; however, a study by Tarasov and colleagues identified a single-nucleotide polymorphism in the gene Col4a1 that was associated with increased central arterial stiffness in humans [33], suggesting that this protein plays an important role in cardiovascular function. The basal lamina surrounding cardiac myocytes contains collagen IV and these studies suggest that the myocyte basal lamina might thicken with age. Interestingly, laminin, nidogen, and perlecan—other significant components of basal lamina—did not exhibit differences in older versus younger tissues. Collagen type IV has also been reported to have a key role in the regulation of angiogenesis as assembly of the basal lamina by endothelial cells is a critical event in new blood vessel formation [34]. *In vitro* studies have shown that collagen IV induces the formation of neovessels, stabilizes neovascular outgrowth, and prevents vascular regression [34]. The increase in collagen type IV, therefore, suggests an increased stimulus for angiogenesis with aging. Whether this results in an actual increase in vessel numbers needs to be evaluated.

Collagen type VI expression was also enhanced with age but differences in expression were not dependent upon SPARC expression (Figure 4). Type VI collagen molecules assemble end to end in a beaded filament arrangement [35, 36]. Typically this collagen is found in close proximity to

collagens types I and III, forming a microfilament network with the fibrillar collagens [37]. Additionally, the N-terminus domain of collagen α1(VI) interacts with the C-terminus domain of collagen IV and in skeletal muscle colocalizes with collagen type IV [37, 38]. These data provide evidence that one of the key roles of collagen VI is to anchor the basement membrane to the underlying connective tissue. Aged-increased deposition of collagen VI may relate to decreased compliance of the left ventricular connective tissue.

Of note, the glycoprotein fibrillin-1 was increased in the middle-aged SPARC null mice. Fibrillin-1 is thought to act as a template for deposition of tropoelastin during elastic fibrogenesis [39]. Moreover, fibrillin-1 exists along individual microfibrils, facilitating their alignment into bundles and interaction with other ECM molecules [39, 40]. Even though fibrillin-1 levels returned to baseline (young levels) in the old null animals, this increase in protein during middle age is suggestive of increased microfibril deposition, which may benefit LV function as the heart ages.

One limitation of this study is that the extraction protocol prevented visualization of collagen degradation products that may occur with age. The decellularization process during the ECM-enrichment protocol removed soluble peptides present in the tissue. Additional studies focusing on the ECM degradome and the effects that these cleavage products may have on cardiac dysfunction with age are warranted.

4. Conclusions

In summary, our data suggest SPARC as a possible therapeutic target for aging induced cardiac dysfunction. Increases in fibrillar collagen with age have been previously reported. We also found age-dependent increases in two other types of collagen expressed in the heart, collagen IV and VI. Although the contribution of these nonfibrillar collagens to diastolic function is currently unknown, increases in collagen IV and VI might influence myocyte interaction with the interstitium. For example, increases in collagen IV might thicken the basal lamina of myocytes impeding extracellular communication between myocytes and fibroblasts and/or myocytes and surrounding vasculature. Collagen type VI has been proposed to influence ECM organization in and around bundles of muscle and vasculature. Hence increases in collagen VI might also affect cell to cell communication or cell to ECM interaction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Proteomic Profiling of the Dystrophin-Deficient *mdx* Phenocopy of Dystrophinopathy-Associated Cardiomyopathy

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Cardiorespiratory complications are frequent symptoms of Duchenne muscular dystrophy, a neuromuscular disorder caused by primary abnormalities in the dystrophin gene. Loss of cardiac dystrophin initially leads to changes in dystrophin-associated glycoproteins and subsequently triggers secondarily sarcolemmal disintegration, fibre necrosis, fibrosis, fatty tissue replacement, and interstitial inflammation. This results in progressive cardiac disease, which is the cause of death in a considerable number of patients afflicted with X-linked muscular dystrophy. In order to better define the molecular pathogenesis of this type of cardiomyopathy, several studies have applied mass spectrometry-based proteomics to determine proteome-wide alterations in dystrophinopathy-associated cardiomyopathy. Proteomic studies included both gel-based and label-free mass spectrometric surveys of dystrophin-deficient heart muscle from the established *mdx* animal model of dystrophinopathy. Comparative cardiac proteomics revealed novel changes in proteins associated with mitochondrial energy metabolism, glycolysis, signaling, iron binding, antibody response, fibre contraction, basal lamina stabilisation, and cytoskeletal organisation. This review summarizes the importance of studying cardiomyopathy within the field of muscular dystrophy research, outlines key features of the *mdx* heart and its suitability as a model system for studying cardiac pathogenesis, and discusses the impact of recent proteomic findings for exploring molecular and cellular aspects of cardiac abnormalities in inherited muscular dystrophies.

1. Introduction

Primary genetic abnormalities in the dystrophin gene result in the early-onset and debilitating muscle wasting disease Duchenne muscular dystrophy or the delayed-onset and milder disorder Becker muscular dystrophy [1–3]. In addition, mutations in cardiac dystrophin are linked to X-linked dilated cardiomyopathy in teenage men [4–6]. A variety of primary or secondary abnormalities in dystrophin-associated proteins are involved in several forms of limb-girdle muscular dystrophy, congenital muscular dystrophy, and dystroglycanopathy [7–9]. The Duchenne type of muscular dystrophy is the most frequently inherited neuromuscular disorder of childhood [10]. It occurs in approximately 1 in 3,500 live born males with substantial regional and national differences in disease frequency [11–13]. Early symptoms of muscular weakness are usually present before 5 years of age and drastically increased levels of serum creatine kinase, pyruvate kinase, and carbonic anhydrase isoform CA3 are characteristic for this type of inherited muscle disease [14–16]. The highly progressive nature of symmetrical muscle wasting often causes a loss of unassisted ambulation around 12 years of age.

Muscle biopsies show an abnormal variation in fibre diameter, large numbers of fibres with central nucleation, necrosis, and a certain degree of regenerating fibres, as well as a progressive increase in fat and connective tissue [10, 20, 21]. In muscle biopsy specimens from Duchenne patients, dystrophin isoform Dp427 is completely or almost completely absent from contractile fibres [22]. In some cases, rare reverting mutants may account for a small percentage of dystrophin-positive muscle fibres [23]. Besides effects on skeletal muscle integrity, abnormalities in dystrophin are also linked to nonprogressive forms of mental retardation [24, 25], scoliosis [26, 27], impaired respiratory function [28, 29], and cardiomyopathic complications [30, 31]. The fact that

respiratory care of Duchenne patients has greatly improved over the years gives the treatment of dystrophinopathy-associated cardiomyopathic side effects a more prominent role in the overall therapy of Duchenne muscular dystrophy [32–34].

This review briefly outlines the pathophysiological significance of cardiomyopathic complications in dystrophinopathies and then focuses on the scientific impact of recent mass spectrometry-based studies of cardiac abnormalities in X-linked muscular dystrophy. Below sections summarize the clinical cardiac symptoms of dystrophinopathy and the pathoanatomical, pathophysiological, and pathobiochemical aspects of the *mdx* mouse heart model of Duchenne muscular dystrophy. Following a brief introduction into the principles of cardiac proteomics as a major biomarker discovery tool for improving our general understanding of cardiac disease mechanisms, recent findings from gel-based proteomic analyses of dystrophin-deficient cardiac tissue and label-free mass spectrometric studies of the aging *mdx* heart are discussed. The considerable influence of cardiac proteomics on the field of muscular dystrophy research and the usefulness of newly discovered proteomic biomarkers for improving diagnostic procedures, prognosis of cardiomyopathic complications in dystrophinopathies, and the evaluation of novel pharmacological or cell-based treatment strategies is examined.

2. Cardiac Dystrophin-Glycoprotein Complex

For a full comprehension of the molecular and cellular complexity of dystrophinopathy, it is important to point out that dystrophin does not exist in isolation within the subsarcolemmal membrane cytoskeleton. Although its overall protein structure and sequence similarity to members of the spectrin-like superfamily of proteins suggest that it possibly forms an intertwined lattice of dystrophin molecules underneath the sarcolemma [35], the linkage to nondystrophin molecules appears to be absolutely vital for sarcolemmal integrity and proper muscle functioning [36–38]. It is well established that the full-length protein product of the dystrophin gene with an apparent molecular mass of 427 kDa forms a supramolecular protein complex at the plasmalemma of both skeletal and cardiac muscle fibres. The core element of the dystrophin-glycoprotein complex consists of the integral glycoprotein β-dystroglycan of 43 kDa that directly interacts on the one hand with the actin-binding protein dystrophin in the subsarcolemmal domain and on the other hand with the extracellular laminin-receptor α-dystroglycan [39]. This large assembly of surface proteins forms a stabilizing linkage between the basal lamina on the outside of muscle fibres and the actin membrane cytoskeleton in the inside of contractile cells [40]. In addition to the core α/β-dystroglycan complex, a large number of additional dystrophin-associated proteins exist, including sarcoglycans, sarcospan, dystrobrevins, and syntrophins [41–44].

Differences exist between the dystrophin-associated glycoprotein complex from skeletal muscle and heart with respect to subcellular localization and protein composition. While the muscle complex is highly enriched in the sarcolemma [45] and at the neuromuscular junction [46], in coexistence with the utrophin-glycoprotein complex [47], the cardiac dystrophin complex is also present in the transverse tubular system [48, 49]. The cardiac dystrophin-glycoprotein complex partially associates with costameric vinculin, suggesting a mechanical role in the maintenance of surface membrane integrity and membrane domain organization [50, 51]. Of note, the recent proteomic analysis of the cardiac dystrophin complex suggests a different range of indirectly associated proteins as compared to skeletal muscle fibres. The cardiac complex appears to lack an interaction with the signaling enzyme nNOS, has a differential composition of syntrophins and dystrobrevins, and displays additional binding partners, including Cavin-1, Ahnak-1, Cypher, and Cryab [52].

3. Dystrophinopathy-Associated Cardiomyopathy

Although dystrophinopathies are primarily categorised as disorders of the neuromuscular system [10], heart disease also plays a crucial role in the etiology of X-linked muscular dystrophy [53]. Almost all patients afflicted with Duchenne muscular dystrophy show clinical cardiac symptoms, especially during the second decade of life [54]. These cardiac abnormalities may include arrhythmias, cardiomyopathy, and regional wall abnormalities [55–59]. A gradual replacement of contractile cardiac fibres by noncontracting cell populations, such as connective and fatty tissue, causes a critical loss of cellular function in the heart of Duchenne patients [55]. The highly progressive decline in the cardiomyocyte population is probably closely connected to the limited regenerative capacity of dystrophin-deficient heart fibres. In contrast to dystrophic skeletal muscles, the heart does not undergo extensive cycles of fibre degeneration and regeneration in dystrophinopathy. In a large number of Duchenne cases, serious cardiac complications result in death [54], warranting special attention to the pathophysiological role of cardiac dystrophin and its associated glycoprotein complex. The primary loss of cardiac dystrophin results initially in changes in dystrophin-associated glycoproteins which in turn triggers a plethora of secondary cellular abnormalities, including sarcolemmal disintegration, necrosis, fibrosis, fatty tissue replacement, and interstitial inflammation. Cellular degeneration leads to progressive cardiac disease and thus fatal complications in Duchenne muscular dystrophy [60].

4. The Cardiac *mdx* Model of Dystrophinopathy

The pathological status of the *mdx* mouse model of Duchenne muscular dystrophy is based on a point mutation in exon 23 of the dystrophin gene, resulting in a truncated protein product that is quickly degraded in dystrophic fibres [61]. Interestingly, different types of muscle exhibit greatly varying degrees of tissue degeneration. While laryngeal, extraocular, and

interosseus muscles show a relatively mild phenotype [62–64] and leg muscles such as *soleus*, *gastrocnemius*, *extensor digitalis longus,* or *tibialis anterior* are moderately weakened by segmental necrosis [65–67], the diaphragm represents the most severely disturbed skeletal muscle type [68, 69] in the *mdx* mouse. Besides the skeletal musculature, the *mdx* heart is also affected by a large number of cellular, physiological, and biochemical abnormalities, as recently discussed in several extensive reviews on the cardiac phenotype of dystrophinopathy [70–72]. Thus, if one takes into account the biological limitations of genetic mouse models as surrogates for human disorders, the *mdx* mouse can be employed as an excellent model system to study basic pathophysiological mechanisms of muscular dystrophy [73].

The dystrophin-deficient heart from *mdx* mice clearly exhibits abnormal histological features, including necrosis, fibrosis, and inflammation [74]. On the subcellular level, a considerable disorganization of the cardiac membrane surface and disruption of the transverse tubular network were revealed by scanning ion conductance microscopy [75]. Signs of overt cardiomyopathy are more pronounced in aged *mdx* mice as compared to milder cardiac alterations in young animals [76, 77]. Aged *mdx* mice showed a widespread and patchy increase in ventricular wall fibrosis [78], whereby the basal region exhibited a greater degree of fibrotic changes than the apex of the dystrophic heart [79]. The onset of fibrosis in the *mdx* heart was found to be associated with an increased expression of collagen and the connective tissue growth factor CTGF [80]. At a later stage of fibrosis, a drastic increase in connective tissue volume was accompanied by the activation of key profibrotic genes, including the heart-specific induction of the Nox4 gene [81]. Coronary endothelial cells are implicated in mediating cardiac fibrosis via transmural TGF-β signaling mechanisms [82]. Interestingly, physical exercise was shown to accelerate the cardiomyopathic process [83, 84]. Exercised *mdx* hearts were characterized by an increase in inflammatory cell infiltration, elevated levels of interstitial fibrosis, and a higher degree of adipose tissue deposition [83]. In the absence of the membrane cytoskeletal protein dystrophin, cardiomyocyte injury was increased considerably by workload-induced cell damage or an acute elevation of mechanical stress [85].

Histopathological features of the *mdx* heart correlate well with the assessment of functional deficits in cardiac output. The dystrophin-deficient heart showed an abnormal electrocardiogram [86] with significant tachycardia and decreased heart rate variability [87]. *In vivo* cardiac MRI studies demonstrated larger right ventricular end-diastolic and end-systolic volumes and lower right ventricular ejection fractions in *mdx* mice [88]. High-resolution doppler echocardiography confirmed that the extent of changes in posterior wall thickness and left ventricular mass are dependent on the age of *mdx* mice [89]. The contractile properties of the *mdx* heart are markedly altered with a reduced force amplitude [90] and considerably prolonged half-relaxation time [91]. The pathophysiological basis of these functional abnormalities is associated with hypersensitive excitation-contraction coupling [92], increased ion fluxes through the fragile plasmalemma [93–95], elevated Ca^{2+}-levels in the cytosol [96, 97], impaired cytosolic and luminal Ca^{2+}-handling [98, 99], enhanced intracellular Ca^{2+}-responses to mechanical challenges [97], an altered mitochondrial redox state, and an increased production of reactive oxygen species [97, 100]. Deficiency in cardiac dystrophin is postulated to cause plasmalemmal fragility, which in turn alters ion fluxes and signaling events at the surface membrane ultimately leading to a pathophysiologically elevated cytosolic Ca^{2+}-concentration [101]. The Ca^{2+}-dependent activation of proteolytic processes and mitochondrial dysfunction probably act as the starting point for the formation of fibrotic patches in the dystrophic heart, as recently reviewed by Shirokova and Niggli [72].

Besides dysregulation of excitation-contraction coupling and Ca^{2+}-handling due to membrane perturbation, metabolic disturbances may predispose the Dp427-deficient heart to contractile dysfunction [102]. Pathobiochemically, the primary loss in cardiac dystrophin isoform Dp427 appears to affect the dystrophin-associated glycoprotein complex in a less severe way as compared to skeletal muscle, possibly due to the upregulation of the dystrophin homologue utrophin [47]. In normal heart, the cardiac-specific dystrophin-glycoprotein complex localizes to the sarcolemma and transverse tubules [48, 49, 103] and probably functions as a membrane-stabilizing linker during excitation-contraction-relaxation cycles in a similar way as the skeletal muscle complex [50, 51], although differences in its composition suggest additional functions [52]. In dystrophy-related cardiomyopathy, both the abundance and glycosylation of α-dystroglycan were shown to be altered in dystrophin-deficient heart muscle [104, 105]. In order to study global changes downstream from the primary defect in dystrophin and secondary alterations in the dystroglycan complex, mass spectrometry-based proteomics was employed for the large-scale analysis of the dystrophic heart.

5. Cardiac Proteomics

Over the last few years, mass-spectrometry-based proteomics has been widely applied to studying cardiac tissues in health and disease. A variety of extensive reviews have been published that summarize and discuss the underlying objectives of cardioproteomic strategies [106, 107], the usefulness of proteomic biomarker research for improving diagnostic, prognostic and therapeutic approaches [108–110], the application of clinical proteomics in the study of cardiovascular diseases [111–113], the evaluation of post-translational modifications in cardiac proteins [114, 115], and technological advances in the field of mass spectrometry and cardiac proteomics [106, 116]. Mass spectrometry-based proteomics was instrumental in the cataloging of the protein constellation of normal heart tissue [117–121], the global assessment of changes in the cardiac proteome during development [122], the determination of functional adaptations following exercise [123–125], and the establishment of protein changes during the natural aging process [126–130], as well as the biomedical analysis of a variety of heart diseases in

patients or animal models of heart disease, including dilated cardiomyopathy, atrial fibrillation, the diabetic heart, and cardiac failure [131–136]. The total number of proteins belonging to cardiac tissues is not known, since no one proteomic method can completely separate and accurately identify all proteins within a complex tissue that exhibits a wide dynamic concentration range. Most likely, the cardiac proteome consists of several thousand different protein species with a wide range of posttranslational modifications [117–121]. For a comprehensive analysis of changes in cardiac proteins with greatly differing physicochemical properties with respect to size, charge, and hydrophobicity, a combination of various proteomic techniques is often advantageous.

Diverse proteomic approaches and methods have been applied in global studies of the heart. For the initial large-scale separation of distinct protein populations, both gel-based and/or liquid chromatography-focused techniques have been employed. Labeling methodology or label-free applications were routinely used for the high-throughput identification of cardiac proteins. Proteomic methods that involve gel electrophoresis are highly suitable for the analysis of contractile proteins, regulatory proteins, metabolic enzymes, metabolite transporters, and molecular chaperones [118]. Two-dimensional gel electrophoresis can conveniently separate cardiac proteins in the range of approximately 10 kDa to 200 kDa and isoelectric points ranging from pH3 to pH11 [117, 118, 120]. Combinations of isoelectric focusing with narrow- or wide-range immobilised pH gradients, native gel electrophoresis, nonreducing gel electrophoresis, and reducing gel electrophoresis can be used for various two-dimensional applications [137–140]. While post-electrophoretic staining with protein dyes is relatively cheap and fast, the differential pre-electrophoretic labeling with fluorescent CyDyes usually results in a larger number of identified cardiac proteins and greatly reduces gel-to-gel variations [141, 142]. One-dimensional gradient gels, in combination with on-membrane digestion protocols, can also cover the separation of high-molecular-mass proteins following detergent solubilization [143]. However, low-abundance proteins, hydrophobic proteins, and components with extreme p*I*-values are difficult to study using routine gel electrophoretic methods [137, 140].

The usefulness of alternative gel-free proteomic labeling methods, such as iTRAQ (isobaric tags for relative and absolute quantitation) or SILAC (stable isotope labeling by amino acids in cell culture), which have been successfully applied to studying cardiac cells [144, 145], has been described in recent reviews [106, 107]. One of the most advanced proteomic approaches involves label-free mass spectrometry. The advantages of this method are that it (i) requires only very small amounts of protein samples, (ii) has broad applicability, (iii) detects a large range of cardiac protein species, and, most importantly, (iv) does not require protein labeling [146]. Thus, in order to overcome some of the problems associated with gel-based methods in cardiac proteomics, label-free mass spectrometry has recently been applied to investigate cardiomyopathic tissue from the aged *mdx* model of Duchenne muscular dystrophy [18].

Figure 1 gives an overview of the key methods employed in comparative cardioproteomic studies and illustrates typical findings from a gel-based analysis of the dystrophic heart proteome. Shown are two-dimensional gels representing the urea-soluble proteome from the young versus the aged *mdx* heart, post-electrophoretically labeled with the fluorescent dye RuBPs (ruthenium II tris bathophenantroline disulfonate) [147]. Fluorescent labeling with RuBPs dye is an excellent and cheap alternative to the more labor-intensive 2D-DIGE approach with its relatively expensive CyDyes [148]. The individual analytical steps performed to achieve the two-dimensional gel image depicted in Figure 1 have been previously described in detail by our laboratory [17].

6. Gel-Based Analysis of Cardiac Changes in Dystrophinopathy

Prior to the development of the proteomic concept and the streamlining of established biochemical techniques for the large-scale analysis of entire protein populations, protein biochemical studies of the dystrophic *mdx* heart have mostly focused on individual proteins, protein complexes, specific pathways, or signalling cascades. Such focused protein chemical approaches, also highly informative about specific aspects of a disease process, inevitably generate biomedical data sets with limited scope. Hence, in order to better complement findings from detailed physiological, cell biological, and histological studies of cardiomyopathic changes, mass spectrometry-based proteomics was used to establish proteome-wide alterations in *mdx* preparations. The parallel analysis of hundreds of cardiac proteins promised to swiftly determine their molecular fate in dystrophin-deficient heart tissues and thus decisively improve our understanding of the molecular pathogenesis of dystrophy-associated cardiomyopathy. Initially, comparative proteomic studies used gel-based surveys of the *mdx* heart muscle and revealed novel changes in proteins mostly associated with mitochondrial energy metabolism, the contractile apparatus, the cytoskeleton, and the cellular stress response [141, 142]. Both studies used fluorescence two-dimensional difference in-gel electrophoresis (2D-DIGE) for the analysis of the dystrophic heart.

The 2D-DIGE technique is an extremely powerful pre-electrophoretic labeling approach that can swiftly determine potential changes in the concentration of thousands of proteins in large analytical gel systems [149–151] and has proven to be an excellent biomarker discovery tool for comparative studies of contractile fibres [152]. The 2D-DIGE method has been widely applied to studying various subtypes of muscle in animal models of Duchenne muscular dystrophy [153–158]. It is one of the key techniques in comparative gel-based proteomics and is employed with fluorescent 2-CyDye [159] or 3-CyDye [160] labeling systems for the differential tagging of proteins from dissimilar mixtures prior to two-dimensional gel electrophoresis [149]. The optimized analysis of 2D-DIGE images with advanced 2D software analysis tools [161–163] can highly accurately quantitate multiple protein samples on the same two-dimensional gel [164, 165]. Importantly,

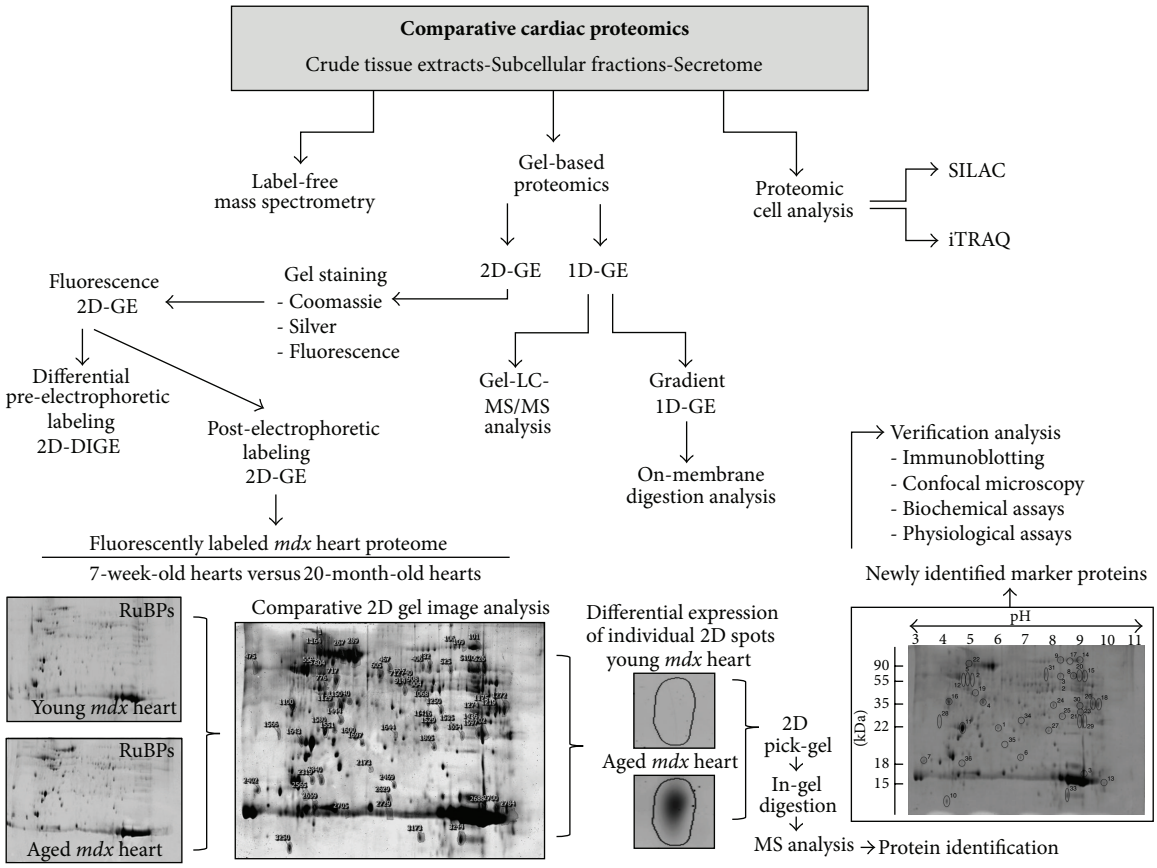

Figure 1: Overview of proteomic methods used in comparative studies of the heart. Shown is a flowchart of the various techniques used to identify changes in the cardiac proteome, including label-free mass spectrometry, gel-based methods (GE, gel electrophoresis), and cellular analyses (SILAC, stable isotope labeling by amino acids in cell culture; iTRAQ, isobaric tags for relative and absolute quantitation). To illustrate the typical work flow of a gel-based analysis of the dystrophic heart proteome, two-dimensional gels representing the urea-soluble proteome from the young versus the aged *mdx* heart are shown. The post-electrophoretic labeling of cardiac proteins with the fluorescent dye RuBPs (ruthenium II tris bathophenantroline disulfonate) was carried out by standard methodology [17].

the completion of reverse DIGE labeling controls is not usually necessary, since selective labeling artifacts were shown not to play a significant role in the analysis of soluble proteins [166], which considerably lowers the overall time and costs involved in large-scale 2D-DIGE studies. The analysis of the murine heart proteome with the 2-CyDye labeling system and the combination of pH 4–7 and pH 6–11 gels resulted in the identification of 2,509 distinct protein spots [142], illustrating the powerful separation and labeling capabilities of the 2D-DIGE technique within the field of gel-based comparative cardiac proteomics [106].

The proteomic profiling of 1-to-9-month-old *mdx* heart extracts by Gulston et al. [141] revealed differential expression patterns for ATP synthase, glyceraldehyde-3-phosphate dehydrogenase, serine proteinase inhibitor, trifunctional enzyme, and hemoglobin. Additional metabolomic analyses suggest metabolic disturbances in the dystrophic heart, agreeing with the altered concentration of key mitochondrial and glycolytic enzymes [141]. Since abnormal heart function was shown to be prominent at 9 months of age [81], a detailed 2D-DIGE analysis of potential changes in the concentration of distinct proteins was carried out with cardiac proteins at this age of *mdx* mice [142]. Electrospray ionization MS/MS analysis identified 26 proteins with a decreased abundance, including various myosin light chains, tropomyosin, actin, adenylate kinase, creatine kinase, vimentin, fatty acid binding protein isoform FABP3, isocitrate dehydrogenase, NADH dehydrogenase, myozenin, porin, and peroxiredoxin. In contrast, 3 heart-associated proteins were found to be significantly increased, including lamin and nucleoside diphosphate kinase. An independent verification of the DIGE analysis was performed by immunoblotting and confocal microscopy of a select group of cardiac proteins. The comparative immunoblot analysis showed a drastic decrease in the enzyme adenylate kinase, the fatty acid binding protein FABP3, isocitrate dehydrogenase, and mitochondrial porin in 9-month-old *mdx* heart tissue [142]. The decreased abundance of the AK1 isoform of adenylate kinase did not correspond with a previous combined metabolomic and proteomic analysis of the *mdx* heart [141] but agrees with several comprehensive proteomic surveys of dystrophin-deficient muscle preparations [152, 153, 167–169]. Since the proteomic result was independently confirmed by immunoblotting, it appears that cardiac nucleotide metabolism that involves adenylate kinase

and creatine kinase is perturbed in the dystrophin-deficient heart.

Mitochondrial dysfunction and accompanied oxidative stress have been linked to various cardiac pathologies, including cardiomyopathy, congestive heart failure, and ischaemia reperfusion injury [170], conveying considerable importance to the results from the proteomic profiling of the *mdx* heart with respect to explaining abnormal mitochondrial function in dystrophy-associated cardiomyopathy [97]. The mitochondrial proteome from heart tissue has been well catalogued and studied using proteomic techniques, focusing especially on the role of mitochondrial proteins in bioenergetics, pathology, and the natural aging process [171–173]. The proteomic finding that a variety of mitochondrial proteins exhibit an altered concentration in the *mdx* heart [141, 142] necessitated microscopical studies in order to evaluate whether these protein alterations were due to a reduced number of organelles in cardiomyopathic tissue or based on internal changes within the mitochondrial proteome. A microscopical survey using the fluorescent labeling of mitochondria with the MitoTracker dye CMXRos, staining of nuclei with the DNA binding dye DAPI, and immunofluorescence staining of cardiac marker proteins revealed no statistically significant differences in mitochondrial content, the number of nuclei, and the subcellular localization of key mitochondrial enzymes between normal and dystrophic heart [142]. Thus, the overall isoform complement of mitochondrial enzymes is not majorly altered, but certain subspecies of distinct cardiac protein isoforms are changed due to the deficiency in dystrophin. Since cardiac mitochondria are the primary site for energy generation via oxidative phosphorylation, even subtle changes in the protein population responsible for oxidative phosphorylation complexes, the citric acid cycle, and metabolite transport can be assumed to have an extensive effect on the bioenergetic status of the *mdx* heart. Besides energy metabolism, cardiac mitochondria are also involved in calcium signaling, the regulation of apoptosis, cell cycle progression, and the production of heme and iron-sulfur clusters [170]. Therefore, alterations in the mitochondrial proteome may affect these crucial cellular functions and render the *mdx* heart more susceptible to damage pathways and ultimately to extensive fibrosis.

7. Label-Free MS Analysis of Cardiac Changes in Dystrophinopathy

Based on the above outlined findings from gel-based proteomic analyses of the dystrophic heart, it was concluded that changes in proteins involved in fibre contraction, nucleotide metabolism, the cellular stress response, mitochondrial bioenergetics, and fatty acid transportation play a central role in the progressive loss of cardiac function in the *mdx* model of Duchenne muscular dystrophy [141, 142]. However, since two-dimensional gel electrophoresis does not properly display very large proteins, these analyses did not produce any information on a key member of the wider network of the cardiac dystrophin-glycoprotein complex, namely, the basal lamina protein laminin. In skeletal muscle, the concentration of laminin is unexpectedly not altered in dystrophin-deficient fibres [40, 152, 174], so it was of considerable interest to determine its molecular fate in cardiac tissue and evaluate whether differences exist in the extracellular matrix of both types of contractile *mdx* tissues. Label-free mass spectrometry suggested itself as an ideal analytical way to study high-molecular-mass cardiac proteins and was therefore applied to determine global downstream effects due to dystrophin deficiency within the cardiac system.

Prior to the proteomic profiling of age-related changes in the *mdx* heart, a label-free LC-MS/MS analysis of 7-week-old dystrophic versus age-matched normal mice was carried out to initially establish potential differences between unaffected and dystrophic heart tissue at an age prior to the occurrence of extensive cardiomyopathic changes [18]. Comparative proteomics established moderate changes in 20 cardiac proteins, which clearly agrees with the relatively mild pathological phenotype in young *mdx* mice. A differential expression pattern was shown for various mitochondrial enzymes, including succinyl-CoA ligase, methylmalonate-semialdehyde dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase, 2,4-dienoyl-CoA reductase, 3-ketoacyl-CoA thiolase, glutamate dehydrogenase, succinyl-CoA: 3-ketoacid-coenzyme A transferase, 2-oxoglutarate dehydrogenase, and isocitrate dehydrogenase.

The detailed proteomic profiling of the aging process in 7-week-old to 20-month-old *mdx* hearts by label-free mass spectrometry demonstrated that aged dystrophic hearts exhibit a generally perturbed expression pattern of key cardiac proteins involved in the stabilization of the basal lamina, the organization of the cytoskeletal network, cellular iron homeostasis, antibody response, fibre contraction, and energy metabolism [18]. Age-related changes were found in 67 cardiac protein species, of which 39 proteins were shown to be increased and 28 proteins were identified as being decreased in their concentration. Of note, the most drastic alterations were increases in transferrin and various immunoglobulin chains and decreases in laminin, nidogen, and annexin. Thus, the collapse of the dystrophin network in the heart and resulting sarcolemmal fragility appears to trigger serious secondary alterations, including the disintegration of the basal lamina structure and cytoskeletal network, an increased level of antibodies in a potential autoimmune reaction of the degenerating heart, and the compensatory binding of excess iron in dystrophinopathy-related cardiomyopathy. Figure 2 shows the bioinformatic STRING analysis of the proteomic data from the recent label-free mass spectrometric study of the aging *mdx* heart. For the evaluation of protein-protein interactions of the mass spectrometrically identified proteins with a changed abundance in the dystrophic *mdx* heart, bioinformatic analysis was carried out with the publically available STRING (http://string-db.org/; version 9.1) database of known and predicted protein interactions that include direct physical and indirect functional protein associations [19]. The interaction map illustrates the enormous complexity of potential protein interactions, especially with respect to mitochondrial components.

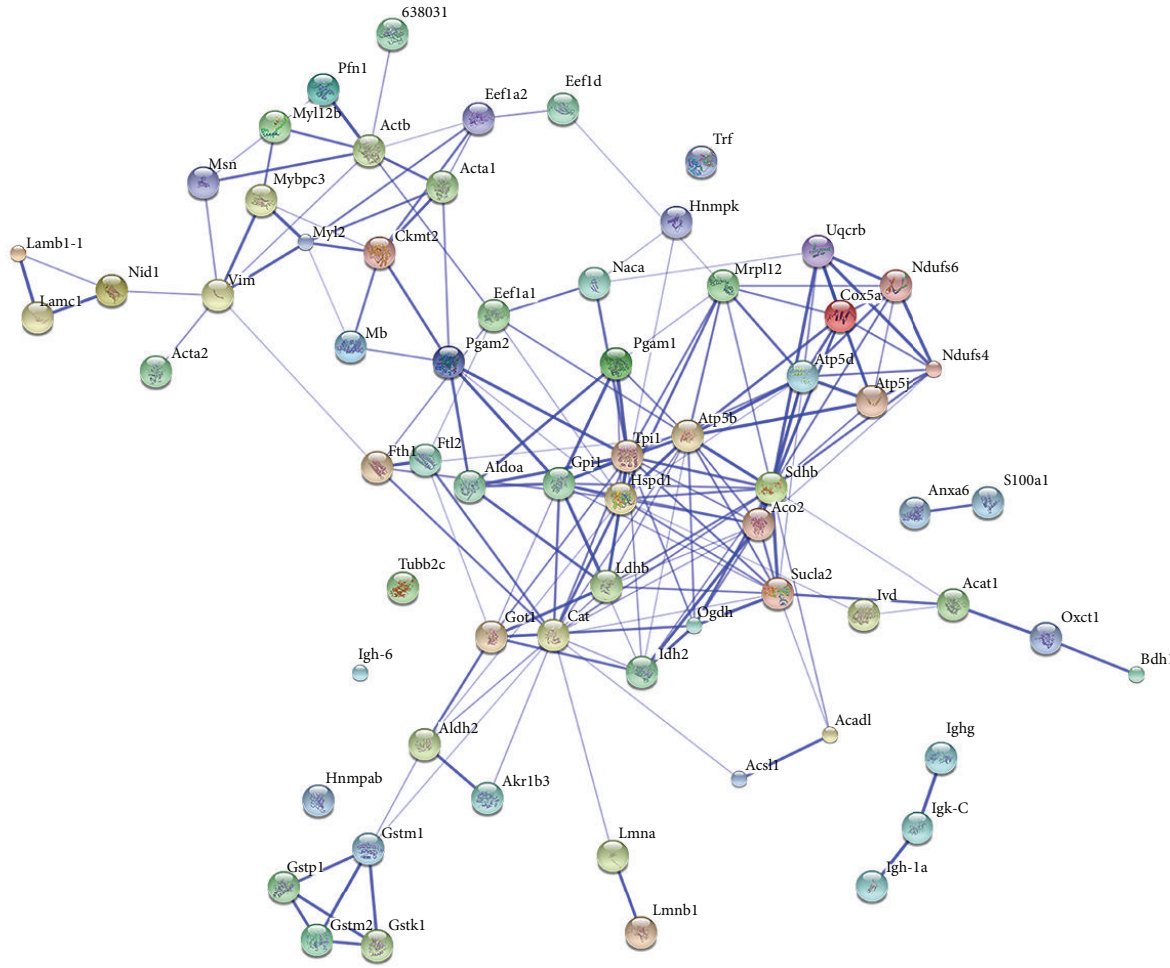

Figure 2: Bioinformatic STRING analysis of the proteomic data from the label-free mass spectrometric study of the aged *mdx* heart. For the evaluation of protein-protein interactions of the mass spectrometrically identified proteins with a changed abundance in the dystrophic *mdx* heart [18], bioinformatic analysis was carried out with the publically available STRING (http://string-db.org/; version 9.1) database of known and predicted protein interactions that include direct physical and indirect functional protein associations [19]. The interaction map of cardiac proteins with a changed abundance in the dystrophic *mdx* heart illustrates the enormous complexity of potential protein interactions, especially with respect to mitochondrial components.

Functional analyses, confocal microscopy, and/or immunoblotting are routinely used to independently verify proteomic data. A comprehensive immunoblot analysis of young and senescent wild type versus *mdx* hearts has verified key proteomic results and clarified differences in protein changes due to natural aging versus muscular dystrophy [18]. While antibody decoration demonstrated that the concentration of laminin, nidogen, and annexin increased during the natural aging process, a drastic decrease in the expression levels of these 3 cardiac proteins was observed in the aged dystrophin-deficient heart. Both, the proteomic data and their confirmation by immunoblotting strongly suggest that the maintenance and architecture of the extracellular matrix, basement membrane, and cytoskeletal network are severely impaired in the aged *mdx* heart. The loss of cardiac dystrophin seems to indirectly affect the essential laminin component of the basement membrane [175] via alterations in the dystroglycan subcomplex. The reduced levels of laminin in turn appear to lower the concentration of nidogen, a sulfated glycoprotein present in many specialized basement membranes [176] and annexin, which is crucial for the maintenance of the cytoskeleton and the extracellular matrix, as well as cardiac Ca^{2+}-homeostasis [177]. This loss in surface integrity of the dystrophin-deficient heart could be one of the major triggering factors that induce progressive fibrosis in dystrophinopathy-associated cardiomyopathy.

The main findings from recent proteomic studies that have focused on the cardiac dystrophin-glycoprotein complex and dystrophin-deficient *mdx* heart tissues are listed in Table 1. The overall emphasis of the individual studies, the main technological approach, and major findings with respect to novel proteomic biomarker candidates of dystrophinopathy-associated cardiomyopathy are displayed. In addition, the flowchart in Figure 3 summarizes the variety

Table 1: Proteomic profiling of the dystrophin-deficient *mdx* heart.

Proteomic study	Methods	Major findings	References
Proteomic analysis of the cardiac-specific dystrophin complex	IP-based copurification, LC-MS/MS, IB, CM	Confirmation of main dystrophin-associated proteins: dystroglycans, sarcoglycans, dystrobrevins, sarcospan, and syntrophins; plus identification of novel dystrophin-associated proteins: Cavin-1, Ahnak-1, Cypher, and Cryab	Johnson et al., 2012 [52]
Comparative proteomic study of 1-month to 9-month-old *mdx* hearts versus age-matched normal hearts	2D-DIGE, LC-MS/MS	Differential expression of ATP synthase, serine proteinase inhibitor, glyceraldehyde-3-phosphate dehydrogenase, trifunctional enzyme, and hemoglobin	Gulston et al., 2008 [141]
Comparative proteomic analysis of 9-month-old *mdx* hearts versus age-matched normal hearts	2D-DIGE, LC-MS/MS, IB, CM	Increased levels of lamin and nucleoside diphosphate kinase; drastic decrease in myosin light chains, tropomyosin, actin, adenylate kinase, creatine kinase, vimentin, fatty acid binding protein FABP3, isocitrate dehydrogenase, NADH dehydrogenase, myozenin, porin, and peroxiredoxin.	Lewis et al., 2010 [142]
Comparative proteomic analysis of 7-week-old *mdx* hearts versus age-matched normal hearts	Label-free MS analysis, IB	Moderate changes in young *mdx* hearts: actin, biglycan, troponin, protein disulphide isomerase, succinyl-CoA ligase	Holland et al., 2013 [18]
Proteomic analysis of the aging process in 7-week to 20-month-old *mdx* hearts	Label-free MS analysis, IB	Severe changes in aged *mdx* hearts: drastic reduction in laminin, nidogen, annexin, vimentin, ATP synthase, cytochromes, NADH dehydrogenase; increases in various IgG molecules, hydroxybutyrate dehydrogenase, ferritin, transferrin, catalase, glutathione transferase	Holland et al., 2013 [18]

Listed are major findings from recent proteomic studies that have focused on the cardiac dystrophin-glycoprotein complex and dystrophin-deficient *mdx* heart tissues. Abbreviations used: 2D-DIGE: two-dimensional difference in-gel electrophoresis; CM: confocal microscopy; IB: immunoblotting; IP: immunoprecipitation; LC: liquid chromatography; MS: mass spectrometry.

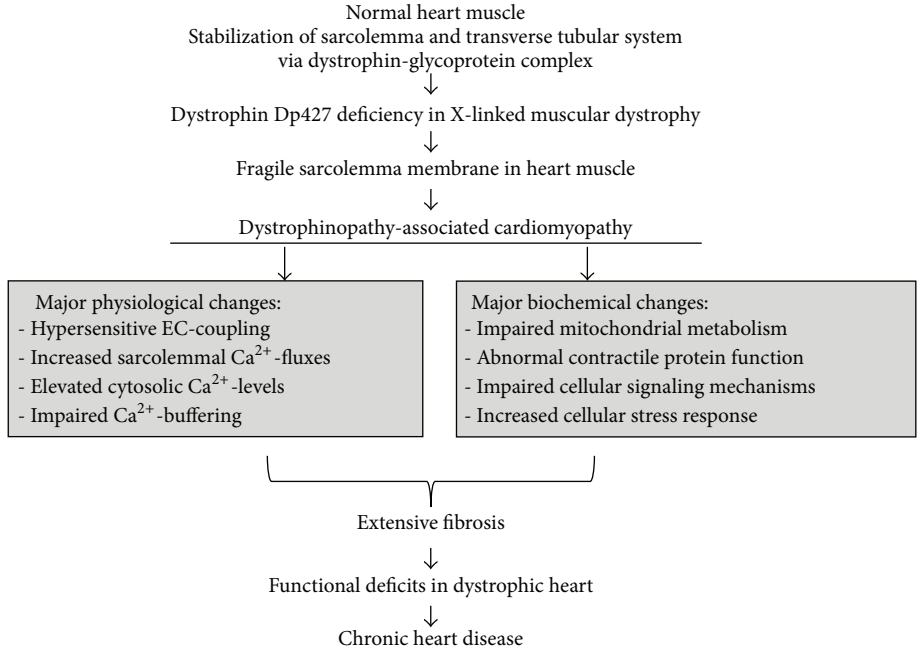

Figure 3: Molecular pathogenesis of muscular dystrophy-associated cardiomyopathy. Shown is a flowchart of major pathophysiological and pathobiochemical changes that render the dystrophin-deficient heart more susceptible to fibre degeneration and fibrosis, which eventually triggers chronic heart disease in dystrophinopathy. Key changes in the physiological regulation of the dystrophic heart are associated with abnormal calcium handling and hypersensitive excitation-contraction (EC) coupling.

of biochemical, physiological, and cellular abnormalities that result in cardiac fibrosis and progressive functional decline of the cardiovascular system.

8. Conclusions

Heart disease is a common clinical manifestation of X-linked muscular dystrophies. Hence, future approaches to treating the overall medical complications present in dystrophinopathy have to take into account the remodeling of incapacitating cardiac fibrosis and resulting functional abnormalities in the dystrophin-deficient heart. As recently reported by Wasala et al. [178], the exclusive correction of abnormalities in the dystrophic skeletal musculature unfortunately does not modulate cardiac pathogenesis in the aged *mdx* model of Duchenne cardiomyopathy. To address this biomedical issue and the fact that a high frequency of cardiomyopathy exists in teenage patients suffering from inherited X-linked muscular dystrophy, a large and diverse number of novel therapeutic approaches are currently tested to specifically address cardiac symptoms in dystrophinopathy. This includes various forms of gene therapy [179–182], exon-skipping therapy [183], and a large number of experimental drug treatments [184–192]. This in turn makes the availability of both a substantial array of reliable proteomic biomarkers and established animal models of muscular dystrophy an important prerequisite for the high-throughput and large-scale testing of new therapeutic options. In order to evaluate the long-term usefulness and potential cytotoxic side effects of gene therapy, exon-skipping, stem cell therapy, and/or pharmacological interventions, simple, cost-effective, and reliable assays with significant protein biomarkers are needed [193].

As outlined in this review, mass spectrometry-based proteomic profiling studies have clearly established the *mdx* mouse as a suitable animal model for exploring molecular and cellular aspects of cardiac pathogenesis and the aged *mdx* heart as a highly appropriate organ system for studying the progressive aspects of muscular dystrophy-associated cardiomyopathy. Most importantly, the application of comparative proteomics has identified a large number of new changes in cardiac proteins associated with cellular signaling mechanisms, mitochondrial energy metabolism, glycolysis, antibody response, iron binding, the contraction-relaxation cycle, basal lamina stabilisation, and cytoskeletal organisation. These novel protein marker candidates can now be used for the systematic screening of the cardiac *mdx* heart following experimental therapeutic interventions. The combined utilization of both label-free mass spectrometry and gel-based techniques promises the most comprehensive coverage of the cardiac proteome, including highly hydrophobic components, low-abundance elements, proteins with extreme isoelectric points, and proteins with extensive posttranslational modifications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Comparison of the Ventricle Muscle Proteome between Patients with Rheumatic Heart Disease and Controls with Mitral Valve Prolapse: HSP 60 May Be a Specific Protein in RHD

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Objective. Rheumatic heart disease (RHD) is a serious autoimmune heart disease. The present study was aimed at identifying the differentially expressed proteins between patients with RHD and controls with mitral valve prolapse. *Methods*. Nine patients with RHD and nine controls with mitral valve prolapsed were enrolled for this study. Two-dimensional difference in-gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were performed. *Results*. A total of 39 protein spots with differential expressions were identified between the two groups ($P < 0.05$, Average Ratio > 1.2 or Average Ratio < -1.2) and four upregulated proteins (including heat shock protein 60 (HSP 60), desmin, PDZ and LIM domain protein 1, and proteasome subunit alpha type-1) and three downregulated proteins (including tropomyosin alpha-1 chain, malate dehydrogenase, and chaperone activity of bc1 complex homolog) were determined. *Conclusion*. These seven proteins, especially HSP 60, may serve as potential biomarkers for the diagnosis of RHD and provide evidence to explain the mechanisms of this complex disease in the future.

1. Introduction

Autoimmunity is the failure of an organism to recognize its own constituent parts as self, thus leading to an immune response against its own cells and tissues. Rheumatic heart disease (RHD) is primarily autoimmune sequelae of acute rheumatic fever (ARF) [1, 2], which occurs after group A beta-hemolytic streptococcal pharyngeal infection [3]. RHD can cause chronic inflammation of the endocardium and myocardium, leading to valvular dysfunction and hemodynamic changes and ultimately resulting in heart failure or stroke and other serious consequences. Due to the lack of a specific means of detection of RHD, many patients have been diagnosed with irreversible valvular dysfunction and scheduled for valvular surgery. RHD continues to be a burden in several developing countries such as India and China, although in the western countries it is reasonably rare probably due to the widespread use of antibiotics [1, 4, 5]. Therefore, an ideal biomarker that can represent the characteristic pathophysiological process of RHD will be valuable for the early diagnosis of this disease, which will help patients avoid surgery by early and effective drug therapy.

Proteomics is the largescale study of proteins, particularly their structures and functions, which enables detection and identification of low-abundance proteins. Proteomics has been extensively used to screen diagnostic biomarkers of diseases such as breast cancer and coliform mastitis [6, 7]. Only one proteomics study of the valvular tissue with RHD was performed [8]. However, no study of myocardium with RHD has been performed earlier.

Table 1: Information of the experimental group and control group.

Group number	Samples	Gender	Age	EF	LVEDD (mm)	Mitral valve stenosis
1	Experimental group	Male	51	0.6	48	Yes
	Control group	Male	51	0.56	54	No
2	Experimental group	Female	49	0.61	45	Yes
	Control group	Female	46	0.59	50	No
3	Experimental group	Female	33	0.68	55	Yes
	Control group	Female	38	0.65	60	No
4	Experimental group	Male	54	0.53	60	No
	Control group	Male	57	0.55	62	No
5	Experimental group	Male	25	0.66	49	Yes
	Control group	Male	30	0.66	47	No
6	Experimental group	Female	21	0.66	43	Yes
	Control group	Female	18	0.63	48	No
7	Experimental group	Female	55	0.56	63	Yes
	Control group	Female	57	0.53	60	No
8	Experimental group	Female	42	0.51	65	Yes
	Control group	Female	47	0.5	67	No
9	Experimental group	Female	43	0.49	55	Yes
	Control group	Female	47	0.51	60	No

In this study, two-dimensional differential in-gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were used to identify the differentially expressed proteins of myocardium in the RHD and the mitral valve prolapse groups. The present study was aimed at identifying the biomarkers, detecting the chronic inflammatory myocardium injury after ARF, and exploring their significance and mechanism in the pathophysiological process of the myocardial lesion in RHD.

2. Materials and Methods

2.1. Sample Collection. The inclusion criteria for the experimental group are as follows: (i) every patient diagnosed as rheumatic mitral valve insufficiency with or without mitral stenosis and scheduled for mitral valve replacement; (ii) normal preoperative erythrocyte sedimentation rate and anti-streptolysin O to eliminate rheumatism in active stage; (iii) all patients in New York Heart Association (NYHA) functional class II-III; and (iv) no other complications; the patients with acute heart failure were excluded. The inclusion criteria of the control group are as follows: (i) every patient diagnosed as mitral valve prolapse because of mitral chordae tendineae fracture and mitral insufficiency and scheduled for mitral valve replacement. The other conditions are the same as criteria (iii)–(v) of the experimental group. RHD cases and their controls were well matched based on the following details: (i) same gender, (ii) difference of age < 5 years old, (iii) difference of left ventricular ejection fraction (EF) < 5%, (iv) difference of left ventricular end-diastolic diameter (LVEDD) < 10% of the larger of the two; and (v) other physiological indexes from physical check in close. Left ventricular papillary muscle was transferred from resected mitral valve to physiological saline and liquid nitrogen and then moved to −80°C refrigerator for storage. Three male and six female pairs totally matched according to the matching principle. The characteristics of pairing groups were presented in Table 1. The study protocol was approved by the Ethics Committee of Ningbo Lihuili Hospital, and informed consent was obtained from all the subjects.

2.2. Sample Preparation for 2D-DIGE. DIGE lysis buffer was added and ground upon ice. The samples were centrifuged and the supernatant was collected to detect the protein concentration. The samples were diluted to 5 μg/μL and the 18 samples were mixed with equal quantity, like 50 μg mixture contains 2.78 μg per sample. The mixture was subpackaged for 50 μg per 10 μL as the internal standard. The pH of the sample was adjusted to 8.0-9.0 for further dye marking. Fifty μg of each sample was labeled with fluorescent dye (GE Healthcare) (the internal standard was labeled Cy2, the experimental group sample was labeled Cy3, and the control group sample was labeled Cy5). The proteins were placed on ice in the dark for 30 minutes for labeling, and finally lysine was added to terminate the reaction.

2.3. 2D-DIGE. The marked samples were combined and sample buffer was added on the ice for 10 minutes. The hydration buffer was added into the labeled samples to a total volume of 250 μL for hydrating the immobilized pH

gradient (IPG) strips (pH 3–10 NL, GE Healthcare), followed by isoelectric focusing (IEF). After IEF, place the IPG strips in the equilibration buffer A and equilibration buffer B, in turn, to reduce the disulfide bonds. For the second dimension, the IPG strips were placed on 12.5% polyacrylamide gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2.4. Images Scanning and Analysis. The individual images of Cy2-, Cy3-, and Cy5-labeled proteins of each gel were obtained using Typhoon FLA9000 imager (GE Healthcare) with the wavelengths of 488 nm (Cy2), 532 nm (Cy3), and 633 nm (Cy5), respectively. The analysis of images was performed through DeCyder 6.5 software (GE Healthcare) to identify the different expression levels of proteins displayed. t-test P value and Average Ratio (control group/experimental group) were used to select differentially expressed protein spots. Protein expression value with an Average Ratio > 1.2 or Average Ratio < −1.2 and $P < 0.05$ was considered to be statistically significant.

2.5. Protein Identification. All the protein spots of interest were selected and excised manually. Sequencing-grade trypsin (Promega, USA) was added for digestion overnight at 37°C and the enzymatic hydrolysate was collected. ZipTip (Millipore, USA) desalination was performed.

The samples were mixed with alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix as a 1 : 1 relationship. The MS and MS/MS data for protein identification were obtained through 4800 Plus MALDI TOF/TOFTM Analyzer (Applied Biosystems). Combined peptide mass fingerprinting and MS/MS queries were performed using the MASCOT search engine 2.2 (Matrix Science, Ltd) embedded into GPS-Explorer Software 3.6 (Applied Biosystems) on the National Center for Biotechnology Information database.

3. Results

2D-DIGE was performed for the nine pairs and 27 maps of 2D gel were obtained (nine maps each for internal standard, experimental group, and control group, resp.). The DIGE images of the left ventricular papillary muscle protein were presented in Figure 1. The distribution and relative intensity of protein spots between groups were consistent. The protein spots in images of RHD mitral valve lesions were compared with those of mitral valve prolapse and 39 differentially expressed proteins were identified as the criterion that t-test P value < 0.05, Average Ratio > 1.2, or Average Ratio < −1.2. The spots with differential expressions were numbered in Figure 2 and their information was presented in Table 2. Of these, 18 spots were overexpressed more in the RHD group than in the control group (Average Ratio < −1.2) and the remaining 21 spots were expressed stronger in the control group (Average Ratio > 1.2).

MALDI-TOF-MS instruments are ideal for protein identification and also for enabling the identification of several proteins in one spot if they are not separated in the electrophoretic procedure. After the incision and enzymolysis of the 28 special spots (failed to identify the other 11 spots) (Table 3), the MALDI-TOF-MS was used to analyze the differential proteins. Finally, 16 spots were successfully identified. The criterion of successful identification was the protein score CI > 95%, while the protein score was > 50. The results were shown in Table 4. There were 10 proteins overexpressed in the experimental group and six proteins overexpressed in the control group. The heat shock protein 60 (HSP 60) (Average Ratio = −3.17) level was more than three times upregulated in the experimental group. With the alpha-actin presenting an equivocal result, more than one alpha-

Table 2: Statistical analysis of abundance difference in differential protein spots (Average Ratio: control group/experimental group).

Number	Spot code	t-test P value	Average ratio
1	34	0.011	1.46
2	36	0.047	1.77
3	47	0.011	1.58
4	57	0.019	1.52
5	66	0.032	1.36
6	68	0.046	1.52
7	109	0.012	1.95
8	127	0.026	1.59
9	137	0.035	1.67
10	532	0.022	−3.66
11	716	0.042	−3.17
12	751	0.00079	−1.92
13	768	0.033	−1.62
14	772	0.021	−1.28
15	847	0.048	−1.53
16	896	0.035	−1.94
17	956	0.0063	1.4
18	974	0.0054	3.37
19	991	0.043	−1.4
20	1017	0.048	1.23
21	1043	0.001	−1.39
22	1057	0.045	−1.38
23	1164	0.034	−1.53
24	1198	0.013	1.24
25	1209	0.0044	1.34
26	1212	0.011	1.24
27	1234	0.032	1.61
28	1271	0.01	−1.77
29	1369	0.017	−1.71
30	1389	0.016	1.36
31	1398	0.023	1.21
32	1410	0.025	−1.52
33	1505	0.048	−1.2
34	1538	0.042	−1.33
35	1713	0.018	−1.39
36	1754	0.0045	1.59
37	1767	0.025	1.31
38	1769	0.048	−1.59
39	2030	0.0089	1.57

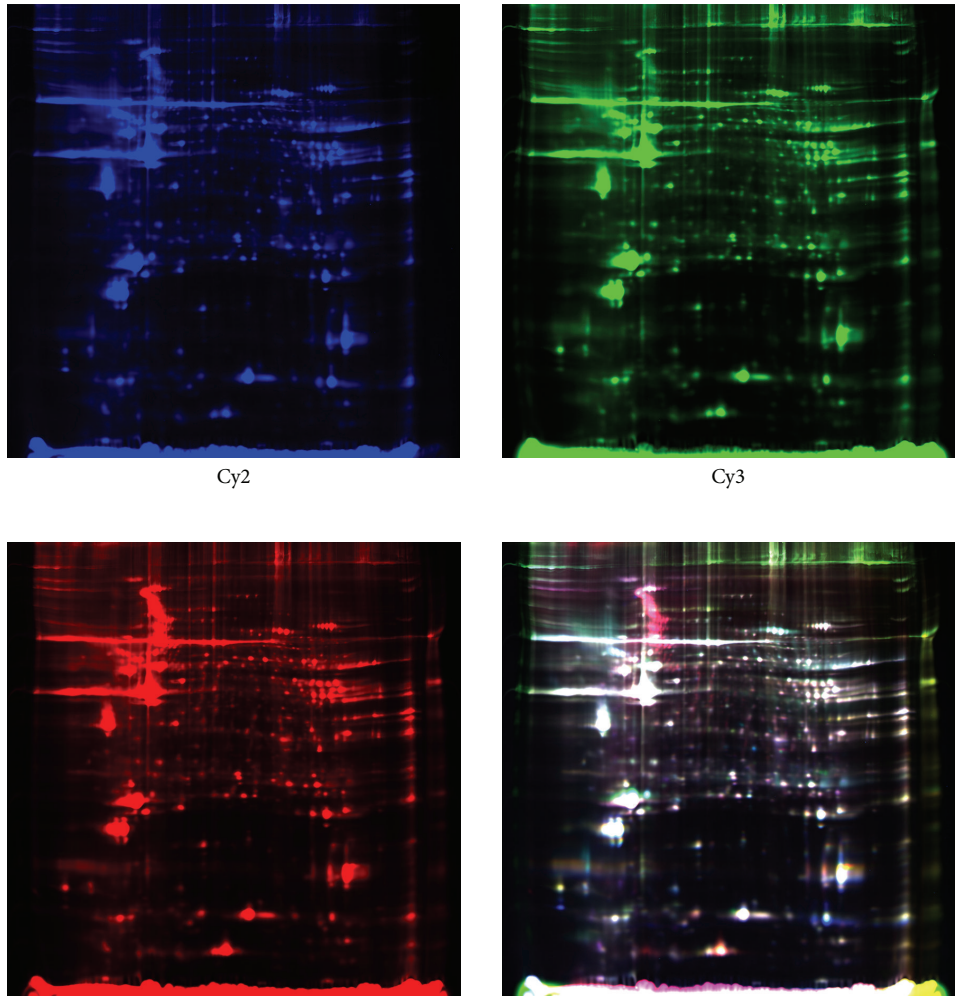

Figure 1: The 2D-DIGE images of left ventricular papillary muscle protein. Internal control samples containing the proteins of experimental group and control group labeled with Cy2 (blue). Proteins of experimental group were labeled with Cy3 (green) while proteins of control group were labeled with Cy5 (red). Color picture was the overlapping images.

actin was upregulated or downregulated in the experimental group (Average Ratio = −1.92, 3.37, 1.23, −1.39, and 1.4).

4. Discussion

Patients with RHD who were scheduled to undergo mitral valve replacement were selected as the experimental group, and patients with mitral valve prolapse were selected as the control group. The two groups were matched in gender, age, EF, and LVEDD to get an exact contrast. Similar EF and LVEDD can eliminate the difference caused by other associated factors such as heart failure. Those proteins whose Average Ratio > 1.5 or < −1.5 were considered to be statistically significant in the difference of protein expression. As a result, 11 differentially expressed proteins were identified. There are seven structure proteins (four types of alpha-actin, desmin, tropomyosin alpha-1 chain, and PDZ and LIM domain protein 1), two zymoproteins (malate dehydrogenase (MDH) and proteasome subunit alpha type-1), and two molecular chaperones (HSP 60 and chaperone activity of bc1 complex homolog (CABC1) protein). The actins were identified in more than one spot due to the great richness of actins in the cardiac muscle. There were four proteins overexpressed in the experimental group (HSP 60, desmin, PDZ and LIM domain protein 1, and proteasome subunit alpha type-1) and three proteins overexpressed in the control group (tropomyosin alpha-1 chain, MDH, and CABC1 protein).

Heat shock proteins (HSPs) are a family of highly conserved, protective proteins expressed in all cells. They primarily protect cells by folding denatured proteins, stabilizing macromolecules, and targeting irreversibly denatured proteins for clearance [9]. However, some findings implied that the released HSP 60 can have a toxic effect on the surrounding cardiac myocytes and lead to apoptosis when myocardium is injured [10, 11]. Extracellular HSP may participate in the inflammatory and autoimmune disorders by activating the innate immune response [12, 13]. As a ligand of toll-like receptor- (TLR-) 4, extracellular HSP 60 can activate TLR-4, which could cause cardiac myocyte apoptosis and inflammatory cytokine production [10, 11, 14]. Intracellular HSP

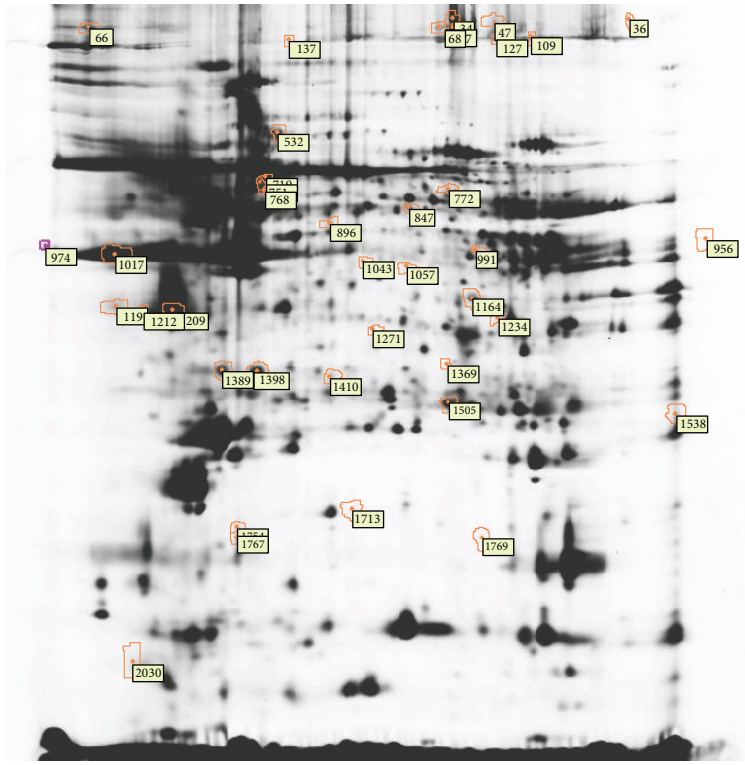

Figure 2: The differential protein spots of RHD left ventricular papillary muscle labeled with number.

60 was released into the media, which also caused cytokine production and TLR-4 overexpression [14]. Although the resected papillary muscle was removed from the serum and pericardium and treated with physiological saline, it was quite difficult to confirm whether the HSP 60 changes detected were extracellular or intracellular considering the innate nature of RHD. It was found that HSP 60 was significantly increased in patients with RHD (3.17 times higher than the control group). However, the overexpression of HSP 60 can activate TLR-4 and potentially stimulate the immune diseases. Although other studies have suggested that HSP 60 levels were increased in the failing heart [15, 16] and the ischemia-reperfusion cardiac muscle [17], the influence of congestive heart failure and ischemia-reperfusion injury has been eliminated in the design of the present study. The unique research about acute RHD and HSP family protein suggested that the HSP 60, HSP 73, and HSP 78 were associated with RHD and the autoimmunity process [18]. It is worthy to observe that in the research the sera from patients with acute RHD were collected as the study sample, while the left ventricular papillary muscle was collected from patients with chronic RHD. The present research indicates the role that HSP 60 plays in myocardial impaired process in RHD more intuitively than sera. Lin and colleagues identified HSP 60 on the surface of cardiac myocytes from failing hearts and suggested that the increased HSP 60 may be deleterious [15]. Other researchers suggested that the increase of HSP 60 may be driven by transcription factor nuclear factor-kappaB (NF-κB) activation [19, 20]. The activated NF-κB can contribute to the immune reaction [21], while the proteasome inhibitor can inhibit the activity of NF-κB [21, 22]. Proteasome participated in the synthesis of active NF-κB. The increased HSP expression can label the misfolding and unfolding proteins for degradation by proteasome [19]. In the present research, the two proteins were both increased in experimental group (3.17 times for HSP 60 and 1.71 times for proteasome subunit alpha type-1). Thus, as the influence of heart failure and ischemia-reperfusion injury has been eliminated, there is a belief that the interaction of HSPs and proteasome may play an important role in apoptosis and inflammation reaction in the myocardium with RHD. This also leads to the inference that the upregulated HSP 60 may be a biomarker for RHD, but certainly further research is required.

Desmin is one of the critical cytoskeleton proteins of cardiomyocytes that will increase due to the myocardial hypertrophy in patients with heart failure [23, 24]. Another two studies pointed out that the myocardial tissue of patients with end-stage heart failure revealed a decrease in or lack of desmin expression [25, 26]. According to Monreal et al. [27], increased desmin expression seems to be a sensitive marker of an early cellular response to mechanical stretch, while the decreased or lack of desmin expression may usually happen in the end stage of some serious cardiac diseases, such as heart failure and idiopathic dilated cardiomyopathy. In the present study, the desmin expression in the experimental group is 1.62 times compared with that in the control group, which we conjectured is because of the longer course of disease and the more significant myocardial hypertrophy in patients with RHD.

Table 3: Information of differential protein spots through MALDI-TOF-MS analysis.

Number	Spot code	Target number	t-test P value	Average ratio	Protein name
1	532	I2	0.022	−3.66	Failed
2	751	I3	0.00079	−1.92	Alpha-actin
3	716	I4	0.042	−3.17	Heat shock protein 60
4	768	I5	0.033	−1.62	Desmin
5	772	I6	0.021	−1.28	Failed
6	847	I7	0.048	−1.53	Failed
7	896	I8	0.035	−1.94	Failed
8	974	I9	0.0054	3.37	Alpha-actin
9	1017	I10	0.048	1.23	Alpha-actin
10	1043	I11	0.001	−1.39	Alpha-actin
11	1057	I12	0.045	−1.38	Failed
12	991	I13	0.043	−1.4	Elongation factor Tu
13	956	I14	0.0063	1.4	Alpha-actin
14	1212	I15	0.011	1.24	Failed
15	1209	I16	0.0044	1.34	Tropomyosin alpha-1 chain
16	1164	I17	0.034	−1.53	PDZ and LIM domain protein 1
17	1234	I18	0.032	1.61	Malate dehydrogenase
18	1271	I19	0.01	−1.77	Failed
19	1369	I20	0.017	−1.71	Proteasome subunit alpha type 1
20	1389	I21	0.016	1.36	Failed
21	1398	I22	0.023	1.21	Failed
22	1410	I23	0.025	−1.52	Failed
23	1505	I24	0.048	−1.2	Peroxiredoxin 6
24	1538	J1	0.042	−1.33	Cysteine and glycine-rich protein 3
25	1754	J2	0.0045	1.59	CABC1 protein
26	1767	J3	0.025	1.31	Failed
27	1713	J4	0.018	−1.39	Collagen type I alpha 1
28	1769	J5	0.048	−1.59	Failed

PDZ and LIM domains containing proteins play diverse biological roles, such as regulation of actin structure, and have been implicated in cardiac and skeletal muscle structure, function, and disease [28–31]. The actinin-associated LIM protein (ALP) subfamily proteins are expressed at the highest levels in skeletal and cardiac muscle [32]. Mouse models and *in vitro* studies suggested that ALP deficiency may influence the development of the right ventricle and ALP enhances the ability of α-actinin to cross-link actin filaments [33–35]. The overexpression of PDZ and LIM domain protein 1 (Average Ratio = −1.53, $P = 0.034$) in the present research may play the role of ALP, which interacts with the α-actinin and enhances the function of actin filament.

Actin and tropomyosin are major components of the actin microfilament system [36]. Tropomyosin is widely distributed in all cell types along the length of actin filaments [37, 38] and regulates the rates of cardiac contraction and relaxation with actin and the troponin complex [39]. In the present research, the decreased expression of tropomyosin α-1 chain (also called α-tropomyosin) in the experimental group (Average Ratio = 1.34, $P = 0.0044$) may influence the relaxation and contraction rate of heart.

MDH catalyzes the conversion of oxaloacetate and malate [40]. The activity of cytoplasmic MDH was decreased with senescence due to shortening of telomere length [41, 42]. It has been reported that cytoplasmic MDH family was significantly decreased in patients with dilated cardiomyopathy by 2D-DIGE [43]. CABC1 is a mitochondrial protein similar to yeast CABC1. The *CABC1* gene, also called *CoQ8* or *ADCK3*, is one of the genes involved in the ubiquinone biosynthesis pathway. A group of *CABC1* gene mutations (R213W, G272V, G272D, and E551K) were identified in ubiquinone-deficient patients with familiar neurologic disease, which caused respiratory-chain impairment and ubiquinone deficiency in muscle tissue [44, 45]. Inhibiting the *CABC1* gene expression partially suppresses p53-induced apoptosis [46]. The association between CABC1 and cardiac diseases was not found. In the present research, the MDH and CABC1 proteins decreased in the RHD group (Average Ratio = 1.61, $P = 0.032$, Average Ratio = 1.59, $P = 0.0045$, resp.). The development of disease may influence the metabolism and cellular processes [47].

The 2D-DIGE experiment is based on fluorescence-based quantitation and the low-sensitivity poststaining may influence the detection. Therefore, numerous low-abundance but differentially expressed dye-labeled proteins may be failed to be imaged. A total of 39 differentially expressed proteins were identified by 2D-DIGE. There were 11 differential spots

Table 4: Information of 16 identified differential protein spots.

Number	Spot code	Protein name	Accession number	t-test P value	Average ratio	PI	MW	Peptide count	Protein Score	C.I.%	Overexpressed in
1	751	Alpha-actin	gi|178027	0.00079	−1.92	5.23	42480	5	111	100	E
2	716	Heat shock protein 60	gi|77702086	0.042	−3.17	5.7	61345.5	9	303	100	E
3	768	Desmin	gi|55749932	0.033	−1.62	5.21	53560.2	31	916	100	E
4	974	Alpha-actin	gi|4885049	0.0054	3.37	5.23	42334	9	207	100	C
5	1017	Alpha-actin	gi|4501883	0.048	1.23	5.23	42381	15	579	100	C
6	1043	Alpha-actin	gi|178067	0.001	−1.39	5.19	37125.3	8	130	100	E
7	991	Elongation factor Tu	gi|704416	0.043	−1.4	7.7	49851.3	16	353	100	E
8	956	Alpha-actin	gi|178027	0.0063	1.4	5.23	42480	15	579	100	C
9	1209	Tropomyosin alpha-1 chain	gi|63252898	0.0044	1.34	4.69	32745.7	22	571	100	C
10	1164	PDZ and LIM domain protein 1	gi|13994151	0.034	−1.53	6.56	36505.2	11	315	100	E
11	1234	Malate dehydrogenase	gi|119620368	0.032	1.61	7.62	31920.5	7	159	100	C
12	1369	Proteasome subunit alpha type-1	gi|13543551	0.017	−1.71	6.15	29864	6	232	100	E
13	1505	peroxiredoxin 6	gi|4758638	0.048	−1.2	6	25133.2	12	427	100	E
14	1538	cysteine and glycine-rich protein 3	gi|4502893	0.042	−1.33	8.89	21867.3	9	387	100	E
15	1754	CABC1 protein	gi|120538499	0.0045	1.59	8.73	44563.7	3	117	100	C
16	1713	collagen type I alpha 1	gi|119615036	0.018	−1.39	5.93	85144.5	9	87	99.947	E

E: Experimental Group; C: Control Group.

that failed to be identified from gel incision. The reasons may be as follows: (i) a portion of the low-abundance proteins were covered owing to the different sampling amounts; (ii) the different coloration methods for proteins caused the difference; and (iii) the samples degraded. Finally, 16 of the 28 spots were successfully identified for which MALDI-TOF-MS experiment was performed. A further western bolt experiment was impossible to perform at this point in time due to the lack of samples from patients with RHD; however, it is known that a confirmed experiment is necessary.

5. Conclusion

In conclusion, in this study, there are seven special proteins found to be significantly different in abundance between the patients with RHD and controls detected through the 2D-DIGE and MALDI-TOF-MS methods. Four proteins, namely, HSP60, desmin, PDZ and LIM domain protein 1, and proteasome subunit alpha type-1, were increased in the experimental group, whereas the other three proteins, namely, tropomyosin alpha-1 chain, MDH, and CABC1 protein, were decreased in the experimental group. HSP 60 may play an important role in the autoimmune pathological process of RHD and could be regarded as a biomarker for RHD. However, this hypothesis needs further confirmation.

Abbreviations

RHD:	Rheumatic heart disease
ARF:	Acute rheumatic fever
2D-DIGE:	Two-dimensional difference gel electrophoresis
MALDI-TOF-MS:	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
EF:	Ejection fraction
LVEDD:	Left ventricular end-diastolic diameter
HSP 60:	Heat shock protein 60
MDH:	Malate dehydrogenase
CABC1:	Chaperone activity of bc1 complex homolog.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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