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Research Article

# Cardiovascular Proteomics – Recent Trends and Strategies

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## ABSTRACT

Cardiovascular disease is the most prevalent class of diseases in the world. Every year more individuals die from cardiovascular diseases than any other illness. Diagnosis and treatment options are limited despite current and past efforts. The molecular mechanisms that lead to the onset and progression of detrimental phenotypes in the heart remain largely elusive. To battle against the ever-increasing number of cardiovascular disease-related deaths, major goals of cardiovascular proteomics studies include the development and utilization of cutting-edge proteomics technologies to map the dynamic cardiac and vascular proteomes, elucidate cardiovascular disease mechanisms, identify candidate therapeutic targets and provide a clinically useful diagnosis as well as risk prediction. Current emphasis is given to promoting the development and adoption of quantitative protein assays targeting highly relevant cardiovascular proteins, such that translation of proteomics technologies may be expedited. Current proteomic approaches in cardiovascular proteomics are discussed.


## INTRODUCTION

Heart diseases resulting in heart failure are among the leading causes of morbidity and mortality in the developed world. They can result from either systemic diseases (such as hypertensive heart disease and is chaemic heart disease) or specific heart muscle disease (such as dilated cardiomyopathy). In most cases of heart failure, the present generation of therapeutic treatments can do little more than ameliorate the symptoms so that, in the majority of cases, the only option is cardiac transplantation. The pathogenic mechanisms underlying cardiac dysfunction in heart failure are still largely unknown, but it is likely that significant alterations in myocardial gene and protein expression underlie the disease processes and determine their progression and outcome. A detailed characterization of these changes will further the understanding of the basis of cardiac dysfunction in heart disease and heart failure, and could provide new diagnostic markers and offer new therapeutic opportunities. Until recently, most molecular studies of cardiac dysfunction have been carried out on specific cellular systems. A recent review of this research describes alterations that have been reported in the expression of contractile proteins, calcium homeostasis and signal transduction [1]. However, researchers in this area have been quick to realize the potential of proteomics to characterize alterations in protein expression in heart disease and heart failure. Such a global approach should provide new insights into the cellular mechanisms involved in cardiac dysfunction.

## MOLECULAR MECHANISMS OF CARDIO-VASCULAR DISEASE

Although traditional proteomics and protein profiling provide important research information, the ultimate goal of developing proteomic techniques for cardiovascular research lies in its ability to characterize molecular mechanisms of disease. In this case, identification of the protein and the nature of its modification are essential. Proteome variations that one might expect depend on characteristics of the particular study being conducted. Altered levels of more than forty proteins have been identified to date in a variety of cardiovascular proteomic experiments (Table). For chronic conditions or disease states, modification of the proteome often manifests itself as altered protein levels due to specific gene up-regulation or down-regulation, isoform switching, or de novo protein synthesis. For example, the abundance of Heat-Shock Proteins (HSP) and mitochondrial and other proteins involved in energy production was documented to vary significantly in a number of heart failure models [2-8]. Isoform switching has also been reported in the case of ventricular expression of atrial MLC1 and MLC2 in neonatal rat myocytes from a phenylephrine-stimulated hypertrophy model [4]. De novo synthesis of HSP72 and two unknown proteins related to the HSP70 family was shown to occur in heat-stressed endothelial cells [5]. In addition, a number of putative PTMs have been documented in dilated cardiomyopathy [2,3,6-8]. For example, detailed 2-DE analysis of dilated cardiomyopathy diseased human myocardial tissue revealed more than fifty HSP27 protein species by immunoblotting [6]. Although only nine were finally confirmed to belong to HSP27 and none were analyzed for the presence of PTMs, this illustrates the potentially large number of PTMs possible for a single protein. In acute conditions, in which there is often insufficient time to recruit de novo transcription and translation, PTMs would be the primary mechanism of protein change, resulting from modification of specific amino acids. To date, cardiovascular proteomic examples of PTM identification are lacking. A prime example of such a study is the identification of phosphorylation and palmitoylation in a membrane-receptor signal transduction cascade [9]. Of course, one should not overlook the possibility of variations in protein levels evinced by protein degradation as a response to acute injury, such as with troponin I in myocardial stunning [10,11] Molecular mechanisms are addressed in one of two ways: by broad-based screening or by a more focused proteomics approach. Broad-based screening is applicable in situations of which little is known of the molecular mechanisms or for an unbiased look at the entire proteome to identify previously unknown protein changes involved in the disease or condition. This extends traditional or protein profiling approaches to the next logical step, identification of the modifications. Cardiovascular examples of broad-based screening include extensive studies on human and bovine cardiomyopathies, as well as many of the studies mentioned above that document changes in protein levels. Focused proteomics, conversely, analyzes only a discrete subproteome. This may be applied to situations in which a molecular mechanism is understood but some components of this mechanism are not. One such example,

**Table 1:** Protein Changes Documented by Proteomic Studies in Cardiovascular Disease.

| Protein Identified | Protein Change | Model |
|---|---|---|
| ATPase synthase | Increase | Human DCM |
| Cytochrome b5 | Decrease | Dog DCM |
| Fattyacid binding protein | Decrease | Dog DCM |
| 3,2-trans-Enoyl-CoA-isomerase | Decrease | Dog DCM |
| Dihydrolipoamide dehydrogenase | Decrease | Dog DCM |
| Isocitrate dehydrogenase | Decrease | Bovine DCM |
| HSP 72 | Novel expression | Heat-shock cells |
| Desmin | Decrease | Dog DCM |
| Actin | Increase | Dog DCM |
| Syndecan-2 | Decrease | Dog DCM |
| Cystatin c | Decrease | Dog DCM |

in cardiovascular research, was carried out by use of Protein Kinase C (PKC) monoclonal antibodies to immunoprecipitated proteins involved in PKC signaling cascades [12].

## NATIONAL AND INTERNATIONAL STRUCTURE OF CARDIOVASCULAR DISEASE

The burden of Cardiovascular Disease (CVD), especially ischemic heart disease and stroke, varies remarkably between regions of the world, with declining rates in Europe, North America, and Australia/New Zealand, burgeoning epidemics in the former socialist economies and India, and relatively lower impact in developing regions such as sub-Saharan Africa. The basis for a prediction of a global CVD epidemic lies in the "epidemiologic transition," in which control of infectious, parasitic, and nutritional diseases allows most of the population to reach the ages in which CVD manifests itself. In fact, CVD is already the leading cause of death not only in developed countries but, as of the mid-1990s, in developing countries as well.

An increased prevalence of coronary heart disease in Asian Indian both from the native country and the immigrant population has been known for some time. With around 15,000,000 Asian Indians living outside India including 1,500,000 in the UK and 1,000,000 in the US, various pathogenic factors have attracted great interest in the recent past. Prevention strategies have been recommended based on these findings. Insulin resistance, central obesity and lipid abnormalities such as high triglyceride levels, low HDL levels and high lipoprotein (a) levels have been documented. This predisposition to accelerated atherosclerosis is genetically determined but is being enhanced by a change in lifestyle, or 'westernization'. An increased prevalence of coronary heart disease because of these changes in lifestyle is seen in India itself, where differences are found between urban and rural populations.

## CURRENT RESEARCH IN THE AREA OF CARDIOVASCULAR PROTEOMICS

### Proteomics of dilated cardiomyopathy

Proteomic investigations of human heart disease have so far concentrated on Dilated Cardiomyopathy (DCM), a disease of unknown aetiology. This is a severe disease characterized by impaired systolic function resulting in heart failure. DCM is almost certainly a multi aetiological disease with known contributory factors being viral infections, cardiac specific auto antibodies, toxic agents, genetic factors and sustained alcohol abuse. The combined results of several studies [13-18] have shown that the expression of approximately 100 cardiac proteins is significantly altered in DCM, with the majority of these proteins less abundant in the diseased heart. Many of these proteins have been identified by chemical methods [15] and they can be classified into three broad functional classes 1. Cytoskeletal and myofibrillar proteins, 2. Proteins associated with mitochondria and energy production, 3. Proteins associated with stress responses. A major challenge will now be to investigate the contribution of these changes to altered cellular functions resulting in cardiac dysfunction. This will require a more traditional system based approach. For example, the expression of Heat Shock Protein 27 (HSP27) in the human heart has been examined in detail. A family of nearly 60 HSP27 protein spots has been demonstrated on large-format 2-DE gels and differences in spot intensity between DCM and controls were observed [6].

### Proteomics of animal models of heart disease

Investigations of human diseased tissues are, of course, complicated by factors such as the disease stage, tissue heterogeneity, genetic variability and the patient's medical therapy. International cooperation, together with improved bioinformatic tools, will be required to investigate the large number of human samples that will be required to overcome these problems. An alternative approach is to apply proteomics to appropriate models of human disease. Such work is still in its infancy, but a recent proteomics analysis of neonatal rat cardiac myocytes treated with phenylephrine (an *in vitro* model of cardiac hypertrophy) revealed 11 proteins with altered expression profiles following induction of hypertrophy [4]. Of these proteins, five have been identified as isoforms of the myosin light chain, two are involved in stress responses and the other four are associated with mitochondria and energy metabolism. There are several models of cardiac hypertrophy, heart disease and heart failure in small animals, particularly the rat. Proteomic analysis of these models has so far been limited, such examples being studies of changes in cardiac proteins in response to alcohol [19] and lead [20] toxicity. A complicating factor of these small animal models is that their cardiac physiology and their normal pattern of gene expression (for example isoforms of the major cardiac contractile proteins) is rather different from that in larger mammals such as humans. Recently, two proteomic studies of heart failure in large animals (pacing-induced heart failure in the dog [21,22] and bovine dilated cardiomyopathy [8] have been published. The results from these studies share similarities with the proteomic analysis of human DCM, with the majority of changes involving reduced protein abundance in the diseased heart. The identification of the altered canine and bovine proteins has been particularly challenging as these species are currently poorly represented in genomic databases, so that new bioinformatics tools have had to be developed to facilitate cross-species protein identification [23]. As for human disease states, changes have been observed in cytoskeletal proteins and proteins associated with the mitochondria and energy metabolism. For bovine DCM, the most notable change was a sevenfold increase in the enzyme ubiquitin carboxyl terminal hydrolase. This could result in increased protein ubiquitination in the diseased state, leading to proteolysis via the 26S proteasome pathway. Interestingly, it has recently been suggested that inappropriate ubiquitination of proteins could contribute to the development of heart failure [24].

### Proteomic characterization of cardiac antigens in heart disease and transplantation

Proteomics is used for both the global analysis of alterations in cardiac protein expression in heart disease, and to identify cardiac specific antigens that elicit antibody responses in heart disease and following cardiac transplantation. In this approach, western blot transfers of 2-DE separations of cardiac proteins are probed with patient serum samples and developed using appropriately conjugated anti-human immunoglobulins. Using this strategy, several cardiac antigens have been identified that are reactive with autoantibodies in dilated cardiomyopathy [25,26] and myocarditis [27]. In addition, cardiac antigens have been characterized that are associated with antibody responses following cardiac transplantation and these might be involved in acute [28] and chronic [29] rejection.

## HEART 2-DE PROTEIN DATABASES

An essential tool for proteomics of the heart is the availability of appropriate 2-DE gel protein databases and, to date, three groups

have established such databases of human cardiac proteins. These databases, known as HSC-2DPAGE, HEART-2DPAGE and HP-2DPAGE are accessible through the WWW and conform to the rules for federated 2-DE protein databases. The databases contain information on several hundred cardiac proteins that have been identified by protein chemical methods. In addition, 2-DE protein databases for other mammals, such as the mouse, rat [13] dog [14] pig and cow, are also under construction to support work on animal models of heart disease and heart failure.

## 2-DE PROTEIN DATABASES ACCESSIBLE VIA THE WORLD WIDE WEB

**Database Web address Organ**

HEART-2DPAGE http://userpage.chemie.fu-berlin.de/ Human heart

HP-2DPAGE http://www.mdc-berlin.de/ Human heart (ventricle)

HSC-2DPAGE http://www.harefield.nthames.nhs Human heart

RAT HEART-2DPAGE http://gelmatching.inf.fu-berlin.de/ Rat heart

## HEART DISEASE AND POST-TRANSLATIONAL MODIFICATIONS

Cardiovascular disease is the leading cause of morbidity and mortality in the developed world. It encompasses various acute and chronic etiologies, including vascular disease (such as atherosclerosis) and heart disease (including hypertension, ischemic heart disease, heart failure and the cardiomyopathies). Cardiac proteins are continuously synthesized and degraded to ensure proteome homeostasis and to generate necessary functions for satisfying the systemic demand for oxygenated blood. The state of the myocardium, which is predominantly made up of cardiomyocytes, dictates the balance between the synthesis and turnover of proteins that ensures that biochemical demands are met. An additional level of regulation involves signal sequences within proteins that mediate folding, trafficking to subcellular destinations and the formation of protein complexes. A still higher-order level of regulation consists of chemical and physical PTMs, and influences the physiological aspects of function and determines the half-life of proteins and their interactions with other proteins, ligands and DNA (Figure 1).

Analysis of PTMs on a large scale has traditionally been difficult because of their generally low abundance [30] and labile nature [31]. Enrichment strategies, such as subcellular fractionation [32] and PTM-focused approaches have now been developed for many PTMs and these have been most successful when combined with the separation power of liquid chromatography and the sensitivity and resolution of Mass Spectrometry (MS). These technologies have now provided a window into the ability of PTMs to decorate proteins. Many of these studies have thus provided vast *in silico* libraries of modified proteins and the sites at which modification can occur [33-39]. Several recent reviews have discussed the use of high-throughput techniques to resolve the contribution of the proteome to vascular disease [40-43].

A diverse collection of enzymatic and reversible modifiers of proteins exists, but for the purpose of this review we will concentrate on phosphorylation, glycosylation (*N*- and *O*-linked) and lysine acetylation.

Phosphorylation is now a well-characterized PTM, with many large-scale studies having been conducted in a wide variety of cell types and tissues since 2006 [44-48]. Over 500 kinases are predicted to be encoded by the human genome, 200 of which have been implicated in heart disease [49-51], as reviewed previously [52,53]. The tools of phosphoproteomics are being used to define the targets of these kinases, including of novel kinases of poorly defined function [54] such as epsilon-protein kinase C, which protects animal hearts from ischemic injury [55]. Hydrogen-peroxide-induced stress in rat cardiomyocytes has also been shown to induce an increase in tyrosine phosphorylation that is mediated, at least partially, by Src kinase [56].

Phosphorylation can alter the conformation of target proteins, resulting in activation or inactivation of function. Current estimates suggest that as many as 500,000 sites can be phosphorylated on between 10% and 50% of the 23,000 proteins in the human genome [57]. Addition of the phosphate group can also recruit (or repel) target proteins, allowing cascades to commence from a single initiator and environmental stimulus. Such signal transduction pathways are crucial in acute cardiac pathologies, such as Ischemia/Reperfusion (I/R), or indeed cardioprotection where the time-frame for injury or protection is too short to be mediated by large changes in protein abundance. Therefore, specific kinase inhibitors (or activators) are of interest as interventions. The specific enrichment strategies used in phosphoproteomics have been reviewed extensively, with a focus on heart disease.

Protein glycosylation, unlike many PTMs, refers to various different modifications of different mass and distribution rather than a single predictable type. Glycosylation can involve the addition of simple monosaccharides through to complex branching glycan structures comprising different individual sugar subunits. Its effects can include alteration or mediation of protein folding, protection against proteolytic degradation, defense against mucosal pathogens,

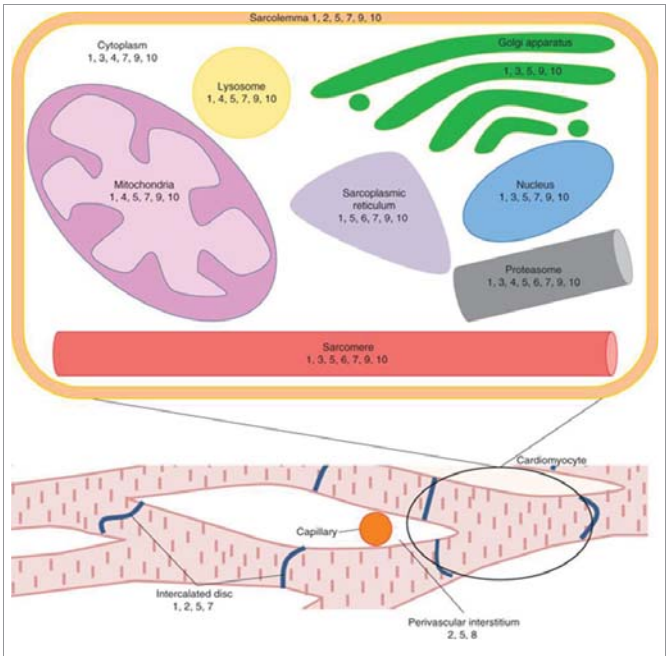


**Figure 1:** Common localizations of specific post-translationally modified proteins within the cardiomyocyte. Post-translational modifications listed are: 1. phosphorylation; 2. N-glycosylation; 3. O-GlyNAcylation; 4. proteolytic cleavage; 5. redox; 6. deamidation; 7. sumoylation; 8. citrullination; 9. methylation; 10. lysine acetylation.

cell-cell communication and adhesion and immunity [58]. Given the number of enzymatic steps required for the addition of sugar moieties, glycosylation is thought to be the most complex PTM, and thus presents particular analytical challenges. Addition of glycans can occur en bloc (in which the glycan is synthesized and added to a previously folded protein substrate) or, more rarely, by sequential transfer of single sugars, one by one, to a nascent (or folded) polypeptide. In both cases, the sugars are attached by oligosaccharyltransferases or glycosyltransferases. Attached glycans can also be modified to add further structural diversity, including sulfation at mannose and N-Acetylglucosamine (GlcNAc) residues in extracellular matrix proteoglycans, addition and acetylation of sialic acid, and phosphorylation, such as on mannose 6-phosphate to ensure correct trafficking to lysosomes [59].

N-glycosylation is a complex biochemical process that is initiated in the cytoplasm, where sugars are synthesized from nucleotide-derived precursors and then transferred into the endoplasmic reticulum, where further glycan assembly occurs. Once the core 14-mer N-glycan is complete, it is transferred by an oligosaccharyltransferase to asparagine residues within the sequence motif Asn-x-Ser/Thr (where × is not proline). Further trimming and maturation, including addition and subtraction of sugars from the core glycan, occurs in the Golgi apparatus. Once complete, the nascent unfolded glycoprotein is generally transferred to the cell surface or extracellular space, and thus analytical techniques for the enrichment of N-glycoproteins are particularly efficient for the capture of the plasma membrane sub-proteome [60]. Changes in N-linked glycans have been observed in cardiac conductance disorders, in heart failure and during remodeling in response to myocardial infarction [61-63]. Typically, however, N-linked glycosylation would not be expected to change significantly in acute cardiac pathologies, and it still remains unclear whether this modification has a role in chronic disease.

Although little work has been done in this area, N-glycosylation of fibrillin-1 has been associated with the genetic defect responsible for the Marfan syndrome, which results in valve and aorta defects [64]. In addition, autosomal recessive dilated cardiomyopathy has been implicated as a congenital disorder of glycosylation in individuals with mutations in dolichol kinase, an essential enzyme in the biosynthesis of glycan precursors [65]. These patients demonstrate N-glycosylation deficiency and abnormal mannosylation of the laminin-binding extracellular matrix protein α-dystroglycan. Additional diseases beyond the classical congenital disorder of glycosylation are now thought to exist [66]. Proteomics, and glycoproteomics in particular, offers the potential to 'type' such diseases by using the diverse chemical properties of the various sugar moieties to determine those absent on a proteome-wide scale in these patients.

Proteomic investigation of mammalian N-linked glycans can be technically challenging given the heterogeneity of the modification and the potential size of the PTM. Enrichment strategies allow the effective capture of glycopeptides [67-69]; however, the labile nature of glycosidic bonds, and the generally large mass of the modification, renders tandem MS approaches problematic. The field is therefore often divided into two: release and study of the diverse glycan structures without reference to the proteins from which they were derived, and release of the glycan and analysis of the formerly glycosylated peptide(s). Key to identifying the site of glycosylation is the presence of the N-linked consensus motif and glycan release facilitated by protein N-glycosidase F (PNGase F), which liberates the N-glycan and 'tags' the former asparagine modification site by deamidation to an aspartate [70].

The analysis of complex O-glycosylation has been comprehensively reviewed recently [71], and little to no information is available concerning a role for complex O-glycans in cardiac diseases [72,73]. Despite this, potentially the best-characterized cardiac-associated PTM is the O-linked attachment of a single β-N-acetylglucosamine (O-GlcNAcylation) to serine or threonine residues of predominantly nucleocytoplasmic proteins. O-GlcNAc thus has the potential to compete with phosphorylation for binding sites [74]. This has led to the proposal of O-GlcNAc-phosphate crosstalk (see below), although there are currently few examples of reciprocal regulation at identical sites and the functional basis (for example, which PTM is the positive and which is the negative regulator of function) for those that have been identified is generally not known.

O-GlcNAc attachment and removal are mediated through the actions of O-GlcNAc transferase and O-GlcNAcase, respectively. Unlike phosphorylation, for which many kinases and phosphatases act either specifically or in large cascades through signal amplification, these two enzymes are currently the only identified mediators of the O-GlcNAc PTM. O-GlcNAcylation has been identified as a PTM of proteins involved in nuclear transport, translation and transcription, cytoskeletal organization, proteasomal degradation, and apoptosis [75,76]. Consequently, it regulates both positive and negative processes in the cardiovascular system [77]. Transient increases in O-GlcNAc provide cardioprotection against myocardial ischemia [78-80] and alter the formation of mitochondrial permeability transition pores during Ca2+ overload [81]. Conversely, the chronic increase of O-GlcNAc observed in diabetes has been linked with adverse cardiac symptoms, including hypertrophy [82] and contractile dysfunction [83]. The physiological distinction between these elevations of O-GlcNAc may be related to interplay with phosphorylation. For this reason, proteomic investigations of O-GlcNAcylation use high-throughput approaches similar to those employed for phosphorylation.

Lysine acetylation is an enzymatic and reversible regulatory PTM that is added by Histone Acetyltransferases (HATs) and reversed by Histone Deacetylases (HDACs). It has been shown to influence gene expression, metabolic processes and chromatin remodeling [84-86]. Irreversible acetylation also occurs on the amino termini of many proteins. A major family of deacetylases is the sirtuins (SIRTs), which are class III HDACs that require NAD for activity [87-89]. The SIRTs have been closely implicated in both protection against and generation of cardiac disease [90-93]. SIRT1 and SIRT7 protect cardiomyocytes from oxidative stress-mediated cell death and age-related degeneration [94]. SIRT2 deacetylase inhibition is protective against I/R injury, because SIRT2 binds Receptor Interacting Protein-3 (RIP3), which is part of a necrosis-promoting complex stimulated by tumor necrosis factor α [95]. Conversely, however, SIRT1 inhibition ameliorates SIRT1-associated cardioprotection against I/R. It is clear that the SIRTs are crucial in cardiac diseases, although the mechanism of this action, and the proteins through which these phenotypes are mediated, remain largely to be determined.

Proteomics approaches based on MS have recently begun to define the extent of lysine acetylation or deacetylation on a systems-wide level [96], revealing a vast level of potential regulation on a scale comparable to protein phosphorylation. Evidence of crosstalk between these modifications through alterations to protein-protein interactions has also been observed [97]. Targets of specific SIRT forms (such as SIRT3 [98]) are now also being investigated such that

both the function of individual SIRTs and their associations with disease can be elucidated. Proteome-wide studies have typically used the specificity of anti-lysine-acetylation antibodies for protein or peptide immunoprecipitation, among other methodologies [99].

## METHODS

Advances in separation sciences have had a particularly tremendous impact on reducing the complexity of peptides prior to the mass spectrometry signal acquisition. High performance shotgun proteomics using mass spectrometry has supplanted Two-Dimensional (2D) electrophoresis to become the de facto standard for large-scale analysis of cardiac proteins (see the general workflow of shotgun proteomics in figure. 2 and figure. 3). Whereas the now-dethroned 2D electrophoresis was limited to detecting a few hundred proteins, contemporary LC–MS experiments can resolve peptides from > 10,000 proteins to allow their identification and quantification.

### Procurement of tissue

Heart tissues from control and diseased human beings are obtained from the hospital. Hearts are rapidly removed within 2-4 minutes after the death of the individuals (normal and diseased) and deblooded in ice-cold 0.9% NaCl solution. Tissue samples (2-4 g each) from the different regions of the heart are freed of fat and connective tissue and are immediately frozen in liquid nitrogen, and stored at -80$^0$C.

### Preparation of tissue samples

All tissue samples are stored in liquid nitrogen prior to processing. The frozen tissue specimens (typically 0.2-0.4 g) are ground to a fine powder under liquid nitrogen using a mortar and pestle. The resulting powder is collected into 1.5 ml microcentrifuge

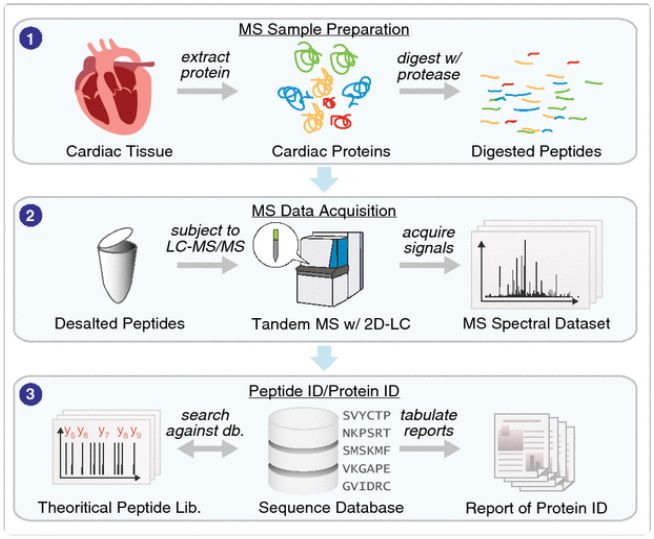


**Figure 2:** Analytical and computational overview in protein identification. 1. Cardiac samples are processed to extract the proteomes or subproteomes of interest, which may then be proteolyzed to obtain peptide digests. 2. The resulting peptides are desalted and subjected to LC–MS/MS analysis to acquire MS1 and MS2 spectra. 3. The peptide sequences that are present in the MS dataset can be identified using a database search approach, which uses a sequence database (e.g., UniProt) to generate theoretical peptide sequence and predict their fragmentation patterns in silico, then automatically find the best-match theoretical spectra to the experimental spectra for protein identification. Alternatively, the proteins can be identified using a spectral library search. The resulting protein datasets can be further analyzed to extract other biomedically meaningful information.

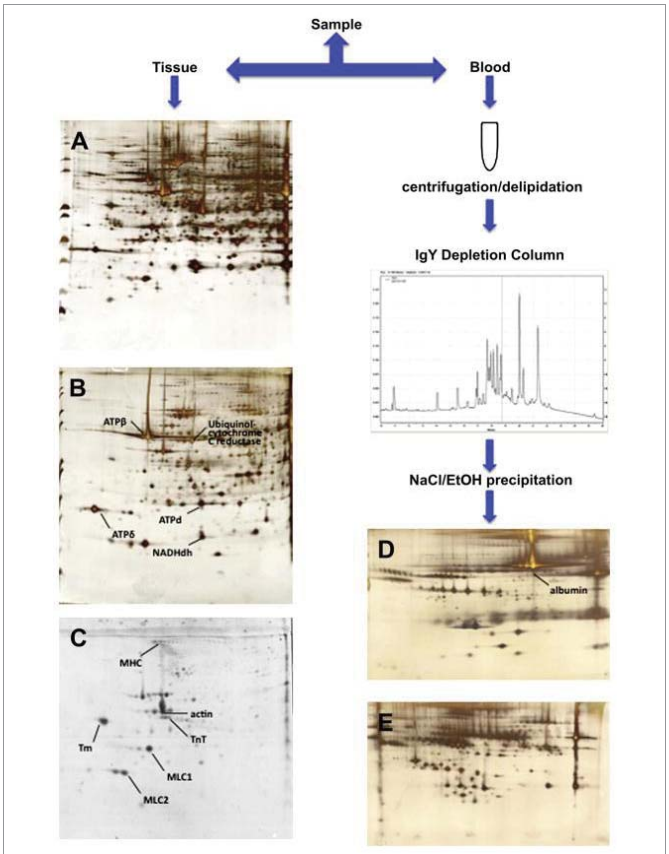


**Figure 3:** Removing the top abundant proteins from the plasma sample is an essential step in enhancing sensitivity and depth of coverage of the downstream techniques. In tissue samples, this can be achieved by enriching the sample for certain subproteomes. Panel (A) shows a silver stained 2D-PAGE image of homogenized cardiac tissue; (B) depicts a silver stained 2D-PAGE image of mitochondria enriched fractions; and (C) shows myofilament-enriched fractions in blue-silver staining (Courtesy of G. Agnetti, Johns Hopkins University). Panel (D) shows a 2D-PAGE image of albumin-associated retenate after depletion of human plasma; whereas (E) shows the protein separation of the depleted plasma itself.

tubes and homogenized using a handheld homogenizer for 1 min in 1 ml lysis buffer, containing 9.5 M urea, 1% DTT, 2% CHAPS and 0.8% pharmalyte pH 3-10. After vortexing for 30 s samples are centrifuged at 15000 rpm for 1 h and the resultant supernatants are collected. Total homogenate protein concentration is measured in duplicate using Bradford method.

### Sub-Proteome fractionation

Prepare the sub-cellular fractions from the liquid nitrogen frozen cardiac tissue. All steps are performed on ice or at 4°C unless otherwise specified. The RCDC assay (BioRad) is used to determine protein concentration during fractionation following manufacturer's recommendations. Heart ventricular tissue (100mg wet weight) is finely diced and homogenized in 8 vol of 20mM $Na_4O_7P_2{\cdot}10\ H_2O$, 20mM $NaH_2PO_4$, 1mM $MgCl_2$, 0.303M sucrose, 0.5mM EDTA, pH 7.0, mammalian protease inhibitor cocktail 1:100 (Sigma) and phosphatase inhibitor cocktail 1:100 (Calbiochem) [100]. The tissue is homogenized (15 strokes) with a 2ml Dounce using both loose and tight pestles (A and B). After the initial homogenization, a sample is collected as fraction 1 or whole homogenate. The whole homogenate is centrifuged in a microfuge at 1000 g for 5 min and the supernatant is saved. The pellet is re-homogenized in 500µL of buffer with pestle

A (ten strokes). The sample is then centrifuged at 1000 g for 5 min and the supernatant is pooled with the previous supernatant. The pellet is resuspended with the aid of a vortex in 500µL of buffer, and then centrifuged in a microfuge at 1000 g for 5 min, repeated once and all four supernatant fractions are pooled [101]. The pellet is kept on ice until enrichment of the sarcomeric, nuclear, and cytoplasmic proteins.

The pooled supernatant fractions are enriched for mitochondrial, microsomal, and some cytoplasmic proteins by centrifugation at 7000 g for 15 min. The pellet is resuspended in half the original volume and centrifuged again at 7000 g for 15 min, and the supernatant is saved for microsomal and cytoplasmic fractions. The pellet contains the crude enriched mitochondrial fraction 3. The pooled volume of the two supernatant fractions are determined, and KCl is added from solid powder to 0.6M final concentration, mix carefully by inversion, incubate for 30 min on ice followed by centrifugation at 142,000 g for 30 min. This step is repeated once. The pellet from the high speed spins is enriched microsomal fraction 4. The two supernatant fractions from the high speed spins are pooled and labeled as fraction 5, which contained mitochondrial, microsomal, and cytoplasmic fractions.

The pellet reserved for enrichment of the sarcomeric, nuclear, and cytoplasmic proteins is re-suspended in 60mM KCl, 20mM MOPS pH 7.0, 2mM $MgCl_2$, 5mM EDTA, 1% (v/v) Triton X-100, mammalian protease inhibitor cocktail 1:100 (Sigma) and phophatase inhibitor cocktail 1:100 (Calbiochem) with 4 volumes relative to the original starting material [102]. The pellet is homogenized using the dounce homogenizer pestle B with at least 15 strokes then centrifuged at 2500 g for 5 min with three repeats. The supernatant fractions are pooled and saved as cytoplasmic containing fraction 6. The final pellet is resuspended and washed twice with 500 µl of 0.1M HEPES pH 8.0. The supernatants are discarded and the final pellet is solubilized in 200 µL of 0.1M HEPES pH 8.0, 8M urea and homogenized with a sonic dismembrator model 100 set at power level 1 (Fisher Scientific). The solubilized fraction is clarified by centrifugation at 20,000 g for 5 min and the supernatant fraction is kept as the enriched sarcomeric and nuclear fraction 2. Analyze the individual fractions further for sub-proteome profiling.

### Two-dimensional electrophoresis

Isoelectric Focusing (IEF) is performed using IPG strips (4-7 and 3-10 pH range). The protein samples are diluted with rehydration solution containing 8 M urea, 0.5% CHAPS, 0.2% DTT and 0.2% pharmalyte 3-10 prior to rehydration overnight. After IEF the strips are equilibrated in 1.5 M Tris pH 8.8 buffer containing 6 M urea, 30% glycerol, 2% SDS and 0.01% bromophenol blue, with the addition of 1% DTT for 15 min, followed by the same buffer with the addition of 4.8% IAA for 15 min. SDS-PAGE is performed using 12%T, 2.6%C separating polyacrylamide gels without a stacking gel.

### Protein visualization

The gels are fixed after electrophoresis overnight in 50% methanol and 10% acetic acid and are stained with silver or Coomassie brilliant blue.

### Densitometry and computer analysis

The silver stained gels are scanned using the calibrated densitometer (Bio-rad) and they are then analyzed using the PD QUEST software (Bio-rad). All gel spots detected as significantly different between the groups are then highlighted and checked manually to eliminate any artifactual differences due to gel pattern distortions.

### In-gel digestion and sample preparation prior to mass spectrometry

In-gel digestion of the Coomassie stained bands is carried out as per standard protocols. Briefly, the gel bands are destained with 50 mM ammonium bicarbonate and 50% acetonitrile solution for 30 minutes. The gel pieces are then dried in a vacuum concentrator and the samples were reduced with 10 mM DTT for 15 minutes and alkylated with 55 mM IAA for 15 minutes. The gel pieces are then dried in a vacuum concentrator and are digested with 12.5 ng/µl of trypsin at 37$^0$C for 18 hours. The gel pieces are then extracted sequentially with 25 mM ammonium bicarbonate and 5% formic acid using a sonicator water bath. These extracts are pooled with the original trypsin supernatant and the sample is dried down in a vacuum concentrator. This is dissolved in 10 µl of 5% formic acid. Silver stained gel spots are first destained with ferricyanide: hypo (1:1) for 5 minutes. The rest of the procedure is same as above.

### Protein identificaction by mass spectrometry

After separation and detection, proteins of interest must be identified. The most significant breakthrough in the evolution of proteomics is the development of MS for protein identification (for review, see References 103-107). Over the past few years, improvements in MS have made it an unrivaled technique, by virtue of its accuracy of mass detection, its detection sensitivity, its ability to deal simultaneously with mixtures of multiple proteins, and its amenity to automation and therefore, high throughput. MS instruments range, in relative terms, from simple (MALDI-TOF) to highly complex tandem (MS/MS). In all mass spectrometers, peptides are ionized from the sample. This is achieved either by Matrix-Assisted Laser Desorption/ Ionization (MALDI) of a solid-state sample or by Electrospray Ionization (ESI) directly from the liquid phase. Ionized peptides are separated on the basis of their mass-to-charge ratio and detected according to their Time-of-Flight (TOF) distribution or analyzed by quadrupole mass filters. In tandem MS/MS, an ionized peptide of interest is selected by the first MS and fragmented by collision with inert gas, and the resulting fragments are then analyzed in the second MS. Modern ESI-based MS/MS may use LC systems such as capillary zone electrophoresis or very low flow–rate reversed-phase HPLC before ionization to fractionate complex peptide mixtures. At the very least, all MS provides precise peptide masses, whereas more sophisticated instruments (particularly tandem MS) also allow peptide sequence determination. A common approach to rapid MS protein identification is peptide mass mapping. Peptide mapping relies on in-gel digestion of proteins by sequence-specific proteases (ie, trypsin, Asp-N, Lys-C) or chemical reagents (ie, CNBr). Since most amino acid residues have a unique mass, protein digestion will yield a set of distinct peptides specific to each protein. A mass spectrum of eluted peptides results, therefore, in a unique Peptide Mass Fingerprint (PMF). The set of peptide masses obtained by MS is then used to search against protein databases created by "*in silico*" cleavage of all known, predicted, or partial protein sequences [108]. The efficiency of this technique is such that it has become commonplace for rapid protein identification [119-111]. For unambiguous protein identification, additional protein sequence information is often required, which can be achieved by tandem MS. A sequence of only five amino acid residues is often sufficient to identify

a protein, unless it is obtained from a highly conserved structural or binding motif. In such cases, additional sequence information will be necessary to narrow down the possibilities and unequivocally identify an unknown protein. As MS instruments evolve, it is hoped that they will all eventually be capable of yielding sequence data, increasing the rigor of protein identification over that of mass fingerprinting.

### Liquid chromatography and mass spectrometry (LC-MS/MS)

The peptide samples can be analyzed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) to discern peptide abundance, isotope incorporation, and sequences. To reduce sample complexity and increase protein coverage, a high-pH/low–pH two-dimensional reversed-phase chromatography can be utilized to separate peptide samples prior to MS/MS [112,113]. The different pH values alter peptide charges to achieve orthogonal separation over hydrophobic stationary phases. First-dimension (high-pH) separation is conducted off-line on a Phenomenex C18 column (Jupiter Proteo C12, 4-µm particle, 90-Å pore, 100 mm × 1 mm dimension) at high pH using a Finnigan Surveyor liquid chromatography system. Establish the solvent gradient profile by mixing solvent A (20 mM ammonium formate, pH 10) and solvent B (20 mM ammonium formate, 90% acetonitrile, pH 10) as follows: 0-2 min, 0-5% solvent B in solvent A; 3-32 min, 5-35% solvent B in solvent A; 32-37 min, 80% solvent B in solvent A; at 50 µl/min – 1 flow-rate. Inject fifty µg of proteolytic peptides with a syringe into a manual 6-port/2-position switch valve. Collect twelve fractions every 2 min from min 16-40, then desiccate in a vacuum concentrator and re-dissolve in 20 µl 0.5% formic acid with 2% acetonitrile prior to low-pH reversed-phase separation. Then perform an on-line second-dimension (low-pH) reversed-phase chromatography on all samples using a single Easy-nLC 1000 nano-UPLC system (Thermo Scientific) on an EasySpray C18 column (PepMap, 3-µm particle, 100-Å pore; 75 µm × 150 mm dimension; Thermo Scientific). Throughout the LC-MS/MS experiment, keep column temperature at a constant 50°C. Inject each high-pH fraction (10 µl) and analyze sequentially using the auto-sampler installed on the nano-UPLC system. Establish the solvent gradient profile by mixing solvent A (0.1% formic acid, 2% acetonitrile) and solvent B (0.1% formic acid, 80% acetonitrile) as follows: 0-110 min: 0-40% solvent B in solvent A; 110-117 min: 40-80% solvent B in solvent A; 117-120 min: 80% solvent B in solvent A; at 300 nl/min - 1. Monitor column pressure to be within approximately 150 bar. Perform high-resolution tandem mass spectrometry (MS/MS) on a single LTQ Orbitrap Elite instrument (Thermo Scientific), coupled on-line to the nano-UPLC system through a Thermo Easy Spray interface. Acquire MS signals were in Fourier-Transform/Ion-Trap (FT/IT) mode: Analyze each FT MS1 survey scan at 60,000 resolving power in profile mode, followed by rapid IT MS2 scans on the top 15 ions with mono isotopic peak selection. MS1 and MS2 target ion accumulation targets are 104 and 106, respectively. MS1 lock mass (m/z 425.120025) and dynamic exclusion (90 s) are used.

### Peptide identification and protein inference workflow

Convert MS2 spectra to .ms2 format using the MS Convert application from the ProteoWizard software package (v.2.1) [114]. Perform Peptide identification using the database search algorithm ProLuCID [115] against a reverse-decoyed protein sequence database (Uniprot Reference Proteome Mus Musculus, reviewed, accessed April-08–2014, 16,672 forward entries and 16,672 decoy entries) [116]. Static cysteine carbamidomethylation (C +57.02146 Da) modification and up to three of the following variable modifications are allowed: methionine oxidation (M +15.9949 Da), lysine acetylation (K +42.0106 Da), serine/threonine/tyrosine phosphorylation (S/T/Y +79.9663 Da), lysine ubiquitylation (K +114.0403 Da), and asparagine deamidation (N +0.9840 Da). Tryptic, semi-tryptic, and non-tryptic peptides within a 20-ppm parent mass window surrounding the candidate precursor mass are searched. Peptide ions from up to 3 isotopic peaks with fragment mass tolerance of 600 ppm are allowed. Protein inference was performed by DTASelect v.2.0 [117], requiring ≤ 1% global peptide false discovery rate and 2 unique peptides per protein for the protein to be considered identified. Modified or non-tryptic peptides are subjected to separate statistical filters to limit false discovery using the –modstat and –trypstat options in DTASelect, such that the inclusion of the variable modifications had no negative impact on the total protein identification counts.

### Protein Quantification

**Stable Isotope labelling by amino acids in cell culture (SILAC):** SILAC makes use of non-radioactive isotope labels to label proteins with light (e.g. 12C) and heavy isotopes (e.g. 13C) [118] Samples can be multiplexed and analysed during the same MS run, thereby minimizing experimental error [119]. The SILAC pairs co-elute during chromatography but the corresponding peptides of the heavy and light isoform appear with a characteristic mass shift. The relative quantity of each protein can be calculated by the differences in the peak intensities of SILAC-labelled peptides. The use of SILAC to quantify differential levels of proteins goes beyond using cells in culture. SILAC-labelled mice have been described with near-complete labelling of all proteins, although the SILAC diet is expensive [120]. Metabolic labelling also introduces information on amino acid synthesis and sourcing, protein assembly, and turnover kinetics

**Isobaric tagging for relative and absolute quantification/ tandem mass tags (ITRAQ):** In instances where human tissue is used, iTRAQ or TMT is an option for multiplexing clinical samples for differential expression studies by LC-MS/MS [121] but these techniques are not without caveats [122]. (i) One disadvantage of the iTRAQ and the TMT system over SILAC is the fact that labelling is performed at the peptide level and occurs late in the experimental process. Before labelling, proteins are first extracted from cells or tissues and digested to peptides. This is a potential source of variation. (ii) Unlike SILAC, quantitation is performed at the MS/MS level, not at the MS level. The peptides from different samples maintain their identical m/z ratios after labelling (MS). Only upon fragmentation (MS/MS), the isobaric mass tags release their different reporter ions with a single isotopic substitution per tag and provide quantitative information for each individual sample. A commonly observed problem in iTRAQ experiments is that a complex background can lead to underestimation of protein fold changes. During precursor ion selection, more than one peptide may be within the mass window selected for fragmentation. In such mixed MS/MS spectra, reporter ions originating from peptides of different proteins are erroneously combined for quantification.

**Label-free techniques:** With advances in data acquisition methods, non-targeted label-free techniques can also reliably deduce accurate protein intensity from shotgun experiments directly through bioinformatics analysis. Label-free quantification is analogous to deducing transcript abundance from reading counts in next-generation sequencing. Existing approaches largely fall into two categories (Figure. 4). Spectral counting exploits the bias of shotgun

proteomics towards abundant proteins, and calculates protein quantity from the stochastic sampling frequency of peptide ions, i.e., the higher the protein abundance, the more of its MS spectra are likely to be identified. A major advantage of spectral counting is that it quantifies directly from the identification output and thus is compatible with most workflows. Spectral counting algorithms tally the number of redundant spectra for each identifiable peptide, then sum the numbers of spectra for all peptides assigned to a protein. On the other hand, ion intensity approaches to integrate the intensity mass-specific ion signals over time in the chromatographic space. This utilizes a feature detection step in data analysis to read raw MS files and integrate the corresponding areas-under-curve of each peptide ion over time. Both labeled and label-free methods provide a useful guide to differential protein expression, and can now be used to discover candidate disease protein that can then be validated by further studies.

## DRUG DISCOVERY AND TARGET VALIDATION

The aim of rational drug development is often to develop new compounds that are able to up- or down-regulate a specific activity involved in disease pathogenesis or in treatment-associated toxicity. It is now apparent that most drugs operate at the level of the protein and that proteins that can be regulated by a drug provide insights into mechanisms of drug action. The proteomic approach clearly has great potential to provide global, holistic analysis of changes in protein expression in response to drug treatment. Modern techniques of HTS are resulting in the rapid discovery of novel, highly active compounds. However, this process does not generally depend on prior knowledge of their structure–activity relationships so that, for many lead compounds, there is no obvious rationale for their activity based on their structure. Proteomics will have an important role both in validating existing drug targets and in providing insights into the pharmacological mechanisms of new drugs derived using HTS. Moreover, it is likely that proteomics will be important in the identification of novel sites of therapeutic action through characterization of complex biochemical events by analysis of pleiotropic alterations in protein expression.

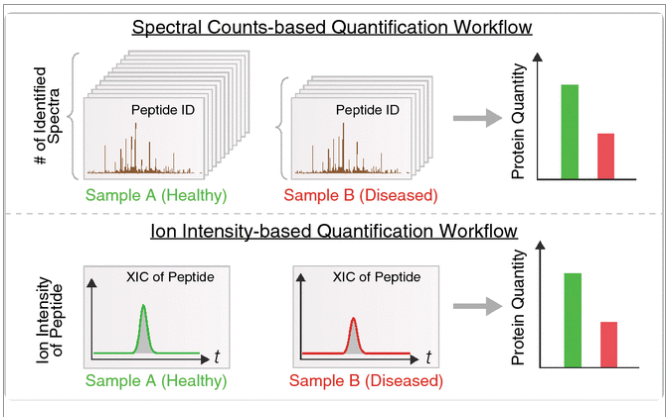


**Figure 4:** Common label-free quantification in proteomics studies. Two common label-free quantification approaches in use are based on spectral count (top) and ion intensity (bottom). Top: spectral counting methods leverage the fact that in stochastic shotgun profiling, the frequency of a protein being sampled by the instrument scales with its relative abundance in the sample. The numbers of spectra matched to an identical protein in healthy (green) versus diseased (red) samples can therefore be compared if appropriate normalization and bioinformatics workflows are implemented. Bottom: ion intensity methods integrate the total signal intensity of peptide ion signals in the mass spectrometer to infer protein quantity. Software is now available to automatically identify and quantify peptide ion signals from mass spectra.

## APPLICATIONS TO CARDIOVASCULAR DISEASE

### Understanding disease mechanism

Unlike the genome, the proteome is dynamic and is much closer to the disease phenotype. Thus, it could lead to a better understanding of the processes responsible for the manifestation and progression of CVD. Currently, proteomic techniques can resolve up to 5000 proteins in a complex biological/ clinical sample, but proteomics platforms are continuously evolving and push the boundaries of analytical instrumentation [123]. In cardiovascular research, the dynamic range in protein expression resulting from abundant myofilament proteins (eg, tropomyosins, myosins, titin) hampers detection of low-abundance proteins in cardiomyocytes. Thus, researchers have developed analytical methods to target specific subproteomes, including cardiac myofilaments [124] cardiac mitochondria [125] cell membranes [126] or nucleus [127]. Researchers recently characterized the remodelling of the vascular extracellular matrix in human abdominal aortic aneurysms, as well as early and late fibrosis in a porcine model of ischemia/reperfusion injury [128,129]. By measuring levels of protein expression without priori assumptions, advanced technologies can help to generate new hypotheses and shift the current focus from quantitative assessment to qualitative differences in cardiac fibrosis that may alter disease progression. The "omics" techniques address the complex interactions within biological systems from a holistic point of view, particularly those interactions involved in the pathophysiology of the disease. Previously, conventional reductionist approaches dealt with intracellular signalling cascades as linear models, with the involved molecules confined to single signal pathways. However, different pathways cross-talk with each other and are organized as networks, including proteins as well as small molecules [130]. Bioinformatics has become an essential tool to comprehensively analyze and visualize these interactions, and databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes) or Reactome map regulated enzymes/metabolites within different metabolic networks [131]. Thus, proteomics provides a read-out to improve confidence in data interpretation [132] and offers a nonbiased suite of tools to interrogate cardiovascular metabolism [133,134]. Atrial fibrillation (AF) leads to several different forms of atrial remodelling, namely electrical, contractile and structural. By using proteomic techniques to analyze the right atrial appendage specimen of patients in sinus rhythm and permanent AF, researchers revealed a potentially novel form of atrial remodeling metabolic remodeling [135]. Several enzymes involved in glucose, lipid, and energy metabolism were altered during AF. The proteomic findings were substantiated by metabolomic analysis, revealing an increase in lipid metabolites and ketone bodies (acetate, beta-hydroxybutyrate). The contribution of metabolic remodelling to AF becoming persistent over time is unknown. However, metabolic alterations appear to precede the onset of AF, suggesting that they are not solely a consequence of AF. In human atrial appendages obtained from patients with and without postoperative AF, dysregulation of cardiac metabolites and altered levels of enzymes relating to energy metabolism occurred before the onset of postoperative arrhythmias [135]. Similarly, the observed proteomic and metabolomic changes in an animal model of an evolving AF substrate (ventricular-tachypacing in dogs) point to the central importance of metabolic changes,

oxidative stress, and associated structural damage in congestive heart failure and profibrillatory changes in the left atrium [136].

## IDENTIFICATION OF BIOMARKER CANDIDATES

The second half of the twentieth century has seen the generalization of minimally-invasive surgical interventions for CVDs, as well as the wider application of a variety of pharmacological molecules such as statins, beta-blockers, antiplatelet therapies, and anticoagulants, which have resulted in an enormous improvement in the outcome and prognosis of patients with CVDs. At the same time, the generalization of rapid, noninvasive standard tests to achieve early detection or risk factor assessment have certainly improved diagnosis and preventive therapies. However, CVDs are rarely attributed to a single factor. In the last few years, it has become apparent that there is a need for new biomarkers for CVDs to better stratify patients and determine their response to therapy. Currently, biomarkers are considered as single entities. In the era of high-throughput technologies, new approaches have become available to multiplex biomarkers for clinical diagnosis and prognostication. Mass spectrometers, for example, are already in clinical use for perinatal diagnostics and toxicology. While immunoassays and biochemical methods have dominated clinical analyses, high-throughput techniques could become more widely used for biomarkers of CVD in the forthcoming years.

## CONCLUSION

Proteomics has contributed significantly to the field of clinical cardiovascular science, both through exploring mechanisms of disease generation and progression as well as by allowing those same processes to be identified, and thus also treated, more efficiently. Proteomics has the potential to reveal those proteins that are associated with pathogenesis. With a greater understanding of information flow in pathogenic situations there will be an increased opportunity for interventions to limit the impact of the disease, and this is an area where proteomics has enormous potential. In addition to the mechanistic contributions of proteomic science, continuing improvements in the field of mass spectrometry, will lead to more effective biomarker discovery and application. This will in turn ultimately lead to increased efficiency of diagnosis and monitoring of treatment, which could dramatically increase the ability of the clinician to recognize cardiovascular disease states at a relatively benign stage, with a concurrent decrease in morbidity.

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