

Proteomics-based Development of Biomarkers in Cardiovascular Disease

MECHANISTIC, CLINICAL, AND THERAPEUTIC INSIGHTS*

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Cardiovascular disease remains a paramount focus of basic science and clinical investigation throughout the developed world, although the demographics have changed considerably in the later half of the 20th century. Atherosclerotic cardiovascular disease, especially ischemic heart disease, has emerged as the major concern, while rheumatic fever and its cardiac sequelae have been superseded by congenital heart disease. Understanding these disease states and their perturbations requires clarification of mechanistic changes in organ phenotype over time, the influence of genetic variations, and the effects of pharmacologic, surgical, and interventional treatment. These requirements have been addressed, in part, by genetic, biochemical, and system approaches. It is clear, however, that knowledge of the DNA sequence, although essential, is not sufficient. A more meaningful understanding of gene expression can be achieved through characterization of the products of that expression, the proteins that are the essential biological determinants of disease phenotype. The ultimate phenotype of cell, organ, and organism is reflected in the instantaneous proteomic profile; similarly changes in human states of health are the result of changes in the proteomes of individual patients over time in response to endogenous and/or external stimuli. The advent of novel proteomic approaches to investigate the complexity of human illness promises to shed new light on the pathogenesis of a broad range of cardiovascular diseases. These inferences are multifaceted and include the commonly recognized role of proteomics in characterizing biomarkers and biosignatures for the prognosis and diagnosis of disease, the capability of these technologies of revealing information regarding functional subproteomes of organelles and networks in the heart and vasculature, and the importance of proteomics in defining changes in these functional subproteomes to guide future therapy.

The central focus of this review is to discuss the importance of proteomics as a non-biased tool for protein discovery. We explore the role of proteomics for hypothesis-driven research into cellular organelle status, and we discuss how this approach facilitates an understanding of the function of the cell in normal and diseased states. Lastly we present a review of proteomics as a tool for biomarker development and lay out a vision for how this technology promises to facilitate distinct biomarker development.

PROTEOMICS AND CARDIOVASCULAR DISEASES

Proteomic Analysis in Diseased Cardiac Phenotypes

Signal transduction pathways are an experimental challenge in the study of complex cell/organ systems. The proteins and the interactions between molecules and post-translational modifications must be studied to understand how inputs into a cell are processed to modulate the phenotype of an organ. Pharmacologic and genetic interventions provide mechanisms by which single molecules can be targeted, thus creating experimental lesions in a signaling pathway that yield insights into the function of its components. Recent investigations in which these types of perturbations have been melded with proteomic analyses of the networks and pathways have afforded novel descriptions of cardiac cell signaling not possible previously.

Importantly the biomedical profession has available an invaluable resource of web-based two-dimensional electrophoretic maps annotated for healthy and diseased cardiac tissues (such as failing myocardium) in a number of extensively characterized species. These resources have recently been highlighted in a comprehensive review by McGregor and Dunn (1). The failing myocardium characteristically displays impaired sarcoplasmic reticulum calcium cycling. It is well established that phospholamban activity is inversely related to the capability of the SR¹ to cycle calcium, and thus an understanding of how this protein modulates the SR subproteome is of critical importance in developing new therapeutic

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¹ The abbreviations used are: SR, sarcoplasmic reticulum; 2D, two-dimensional; PTM, post-translational modification; HSP, heat shock protein; PKC, protein kinase C; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; 2-DE, two-dimensional gel electrophoresis; SMC, smooth muscle cell; EPC, endothelial progenitor cell; CRP, C-reactive protein.

strategies that aim at modulating the performance of the failing heart at the molecular level. Chu *et al.* (2) used classical 2D electrophoresis and mass spectrometry to examine protein changes in hearts from phospholamban knock-out mice as compared with wild type controls. This approach unveiled numerous alterations in myofibril proteins and in calcium-handling proteins, including evidence of altered PTMs. These alterations in diverse sets of proteins were initiated by removal of phospholamban and are potentially related to enhanced cardiac performance in the genetically altered mice. An understanding of how this protein modulates the SR subproteome is of critical importance in divulging therapeutic strategies that modulate performance of the failing heart at the molecular level. In another example of coupling genetic modulation of individual molecules to proteomic signaling pathways analysis, Buscemi *et al.* (3) elegantly examined the proteomic changes concomitant with Rac1 activation in the heart. By focusing their analyses on the myofilament and cytoplasmic organellar subproteomes, these investigators were able to decipher 12 changed proteins by 2D electrophoresis, including quantitative differences in tubulin, manganese-superoxide dismutase, and malate dehydrogenase in the early stages of development of the dilated cardiomyopathy phenotype (within the first 2 weeks after birth). These analyses did not reveal changes in PTMs in the myofilament subproteome. Such insights would not have been possible with the genetic perturbations in isolation. Proteomics was required to discover these aberrant signaling mechanisms.

Several crucial investigations have explored the proteomic nature of altered cardiac function and growth using non-genetic models. Pulmonary artery banding was used to induce an increase in right ventricular mass in rats, and changes in cytosolic proteins were examined by 2D electrophoresis and mass spectrometry (4). Interestingly the study revealed changes in cytosolic heat shock proteins, and subsequent quantitative analyses with immunoblotting determined that these stress molecules were phosphorylated in the hypertrophied right ventricle. Because of the ubiquity and necessity of heat shock proteins for stress signaling in the heart (4–7), it is not surprising that other proteomic studies utilizing distinct models of cardiac stress signaling observed alterations in heat shock protein expression and modification. Changes involved α B-crystallin and HSP27 in a tachycardia-induced model of congestive heart failure in dogs (5) and alterations in multiple HSPs (potentially including phosphorylation) in response to oxidative stress in neonatal rat cardiomyocytes (6).

Proteomic studies can serve as necessary precursors of focused biochemical and physiological analyses designed to confirm functionality within the signaling pathways. Although proteomic analyses of the protein kinase C ϵ (PKC ϵ) signaling subproteome had identified many of the molecular partners of this protective kinase, the specific manner in which these proteins associated with PKC ϵ and their intracellular tasks remained unknown. Subsequent biochemical and physiolog-

ical functional assays premised on these proteomic results were effective in delineating novel roles for PKC ϵ in modulating endothelial nitric-oxide synthase and GSK3 through Akt signaling (8) and in modulating mitochondrial permeability transition in cardiac mitochondria (9). These insights were facilitated by a proteomic map of the pertinent signaling pathways involved in the phenotype.

Proteomic investigations also related to mitochondrial signaling in the myocardium have been used to reveal alterations in mitochondrial signaling mechanisms in different cardiac phenotypes (9–13). Proteomic analysis of ischemic/reperfused rabbit hearts (examining whole cell lysates, cytosolic fractions, and myofilament compartments) elucidated gel shift and protein abundance changes via 2D electrophoresis in multiple proteins, particularly those associated with stress response (see above) as well as energy metabolism in mitochondria (12). These studies highlight the utility of proteomics for rapid changes in signaling pathways, such as those occurring during myocardial ischemia/reperfusion, which might not be detected by gene-based analyses. Aberrant signaling in mitochondrial function was also observed in the aging monkey heart (13) in which sex-specific differences were uncovered relating to the propensities of these animals to develop age-related cardiomyopathy. Interestingly the mitochondrial subproteomic alterations correlated with susceptibility to injury, suggesting that these changes in mitochondrial signaling may also serve as markers of disease and reflect mechanisms of injury (13). In focused analyses of mitochondrial respiratory complexes (11), proteomic analyses were used to divulge the molecular participants in signaling at these critical regulators of mitochondrial function and cell death/survival. Immunoprecipitation and proteomic identification mapped the subproteome of molecules interacting with these members of the mitochondrial subproteome, paving the way for future functional analyses of how signaling pathways modulate mitochondrial function and permeability transition.

Together these studies illustrate how proteomics permits the interrogation of specific cellular networks in the heart while unveiling in a non-biased manner the mechanisms of signal propagation by studying post-translational modifications. Such global insights into cellular pathway makeup and function would not have been possible with solely genetic and pharmacologic approaches; in essence, a functional proteomic approach facilitates an understanding of signaling pathways in the heart.

Vascular Proteomics

Compared with other areas of proteomic research, vascular proteomics is still in its infancy. Only a few publications applying proteomics to the vasculature have been published, and the proteome of vessels of different sizes is largely unknown. A major obstacle to the proteomic analysis of vessels is their heterogeneous cellular composition. Most attempts to

apply proteomic techniques to human atheroma have been limited by the presence of serum proteins and the biological variation in human lesions (14–18). Different strategies have been applied to overcome this limitation, including dissecting the media from human vessels (19) or *ex vivo* culture of vessels in protein-free medium to analyze secreted proteins in the supernatant (17). Moreover with the introduction of apoE-deficient and low density lipoprotein receptor-deficient strains, the mouse became the preferred animal model in cardiovascular research (20–22), offering the opportunity to analyze protein changes during various stages of atherogenesis under well defined laboratory conditions and in animals with identical genetic backgrounds, thereby facilitating proteomic comparisons by limiting biological variation. Notably when we compared 2D gel protein patterns of apoE^{-/-} aortas classified according to their atherosclerotic surface area in vessels with mild (<10%), medium (10–30%), and severe atherosclerosis (>30%) (23), only a few of the most abundant macrophage proteins, *e.g.* CapG, which account for 0.6% of the total, showed a detectable increase in advanced stages of disease. The bias of 2D gels toward high abundance proteins can be advantageous because proteomic profiles of tissues with heterogeneous cell composition remain dominated by their most abundant cellular components, facilitating proteomic comparisons during disease progression. An alternative to studying blood vessels *per se* is the use of cultured cells resembling phenotypes present in atherosclerotic lesions, but it must be considered that vascular cells are designed to respond to mechanical forces *in vivo*, whereas cell cultures necessarily represent artificial conditions associated with dedifferentiation and altered protein expression (24, 25).

Significant work has not yet been done on mature endothelial cells (ECs) derived from large veins and arteries. Human umbilical vein endothelial cells (HUVECs) derived from transient fetal vessels are currently the most popular *in vitro* models. Two annotated 2-DE maps (26, 27) have been made available. There has been work on dermal microvascular endothelial cells (28, 29) and in the human endothelial cell line EA.hy926 (30, 31) generated by fusion of HUVECs with the human lung carcinoma cell line A549 (32).

The term smooth muscle cell (SMC) is used within the vasculature to include any connective tissue cell that forms a coating around the endothelial tube (33). In contrast to ECs, SMCs in different parts of the vascular tree develop from different germ layers. For example, the descending aorta and most muscular arteries including the coronary arteries contain SMCs derived from the mesoderm, whereas the SMC compartment within the pulmonary trunk, the aortic arch, and the ascending aorta originate from the neuronal crest (ectoderm). Hence a great variation has been observed among different SMC populations (34, 35). Moreover SMCs can acquire macrophage-, adipocyte-, chondrocyte-, and even osteocyte-like phenotypes (36–39), whereas ECs can delaminate from the monolayer and transdifferentiate to SMCs (40). Given

this astonishing plasticity within the vascular system, proteomics may be useful in obtaining a better understanding of cell differentiation and in progressing toward a molecular classification based on protein expression patterns of vascular cell types. In a first attempt, we have recently analyzed the proteome of Sca-1⁺ progenitor cells, the early differentiation step from embryonic cells to SMCs, and compared their proteome with differentiated mature SMCs (23, 41). A comparison of all SMC maps published so far is available (19, 42). Reviews of current literature in proteomics on the two most prevalent phenotypes of vascular diseases are provided below. Completely unexplored are the abnormalities of elastin, smooth muscle, and collagen that characterize dilated great arterial walls in a wide variety of congenital heart diseases from the neonate to older age (43) and in the media of the dilated tortuous extramural coronary arteries of patients with cyanotic congenital heart disease (44).

Atherosclerotic Cardiovascular Disease

According to the World Health Organization definition, atherosclerosis is a variable combination of intimal alterations consisting of foci of lipid accumulation, complex carbohydrates, blood and blood components, connective tissue, and calcium deposits, which are linked to alterations of the arterial media. Atherosclerosis is a complex process with different risk factors acting in its various stages. The disease is generally assumed to emerge from an interaction between injurious or protective environmental influences and the genetic background of given individuals.

Endothelial injury is the key event that precipitates the atherosclerotic process (45–47), and inflammation has assumed a prominent role (see additional discussion later). Endothelium lines the inner surface of all blood vessels covering an area of ~1000 m² and amounting to a total weight of 1 kg in humans. Endothelium forms a selective barrier between blood and tissue, providing an antithrombotic surface, inhibiting leukocyte adhesion and smooth muscle proliferation, and regulating vascular tone via synthesis and elaboration of nitric oxide. Cardiovascular risk factors, including elevated serum cholesterol, systemic hypertension, diabetes mellitus, smoking, and infectious agents, lead to endothelial injury with loss of antithrombotic and anti-inflammatory properties, compromise of barrier function, increased trapping of lipoproteins in the arterial wall, and formation of reactive oxygen species that decrease the bioavailability of vasodilator nitric oxide and cause paradoxical vasoconstriction, a hallmark of endothelial dysfunction (48).

The first recognizable atherosclerotic lesion, the “fatty streak,” comprises an aggregation of lipid-rich macrophages within the intima (49, 50). Advanced lesions contain large numbers of monocyte-derived macrophages with cell viability compromised by the formation of large necrotic cores. Rupture of the necrotic core within an atherosclerotic plaque

disrupts the endothelial surface and induces clot formation and myocardial infarction (51).

The classic concept of atherosclerosis proposes that neighboring endothelial cells serve to re-endothelialize sites of vascular injury and that local SMCs together with macrophages and lipids form atherosclerotic lesions. There is now increasing evidence that circulating endothelial progenitor cells (EPCs) contribute to vascular repair by maintaining endothelial integrity and that SMCs within atherosclerotic lesions are not only derived from the local media but also from progenitor cells (52–55) whose precise origin remains subject to controversy (56, 57). Importantly the number of circulating EPCs correlates with endothelial function (58), is reduced in subjects with vascular risk factors, and predicts the risk of future cardiovascular disease (59). Hence the assessment of EPC numbers might prove to be a useful biomarker of cardiovascular risk. Despite decades of research, there is still no agreed upon pathogenetic hypothesis that traces the sequence of events between the development of the fatty streak and advanced atherosclerosis (60). Promising emerging technologies prefigure a more comprehensive understanding of the complex pathophysiological disease processes underlying atherosclerosis. The negative relationship between coronary atherogenesis and the hypoxemia and erythrocytosis of cyanotic congenital heart disease has only recently come to light (61).

Hypertension

Elevated blood pressure (systemic hypertension) is one of the most common diseases in the United States and is responsible for heart attacks, heart failure, strokes, and chronic renal disease. Despite long and vigorous research efforts and despite the progress in understanding the genetic basis of hypertension, its cause(s) remains largely unknown. Some simple but rare Mendelian forms of hypertension have been characterized in humans, but these forms account for no more than a very small percentage of cases (62, 63). Familial and association studies have identified regions of the genome with alleles associated with risk of hypertension, but these regions often contain hundreds of genes and regulatory regions that make it difficult to isolate causative factors (64). Proteomic approaches provide opportunities to link gene products to function by specifically analyzing the levels of proteins generated. These data can then be used to validate the results of genome scans. The still unresolved pathogenesis of hypertension associated with coarctation of the aorta (65) also lends itself to proteomic analysis.

Another type of study that often implicates a gene or set of genes as causative for a disease is an association study in which a single nucleotide polymorphism or a set of polymorphisms is correlated with a higher than expected probability of having the disease. It is often the case in these studies that the mutated gene simply resides near the gene responsible

for the disease rather than being responsible itself. Proteomic approaches, in which the proteins involved are measured as an intermediate phenotype, can help to resolve such results and support the hypothesis that a specific gene is causative (66).

Animal Models—One model in which proteome analysis has nicely complemented genetic research strategies is derived from consomic rat lines in which cohorts are developed and maintained from two different strains in which each chromosome from one strain has been introgressed into a rat with the other genetic background (67). These consomic rat lines show significant variation in phenotype (e.g. blood pressure). Analysis of plasma, urine, and endothelial cell-derived proteins isolated from parental and consomic rat strains allows quantitative examination of the proteome by standard tandem mass spectrometry using isotopic labeling with ^{18}O (68). The use of consomic rat strains offers a unique resource for the systematic study in closely related animals of genetically complex diseases such as hypertension, thus reducing the complexity of the proteomic differences.

In Vitro Models—Proteomic analysis of *in vitro* systems can add directly to our understanding of cardiovascular diseases.

A variety of studies can be performed in which specific proteins or enzymes linked to hypertension are regulated at the molecular level in cell culture models. Using MS quantitation methods such as ICAT for determining relative protein and phosphoprotein levels in cell lines, potential pathways relevant to the diseases can be identified (69). This proteomic approach has the capacity to rapidly identify elements of unknown signaling cascades and to provide important new insights into the molecular mechanisms underlying human hypertension. As a result of proteomic analysis, members of critical pathways might be found to represent new targets for development of novel antihypertensive agents.

Developments in proteomic technologies and analyses should permit more complete characterization of the cellular proteome with the potential of complementing genetic studies and providing a unique set of insights not possible with DNA or RNA based methods *per se*. Improvements in our understanding of protein modifications and interactions will open new diagnostic and therapeutic approaches to the management of cardiovascular disease.

In addition to changes in protein expression and protein interactions, recent investigations in vascular signaling have highlighted the importance of altered PTMs on the function of vascular cell proteomes. In a recent study by Handy and Loscalzo (70), the importance of S-nitrosation (a reversible PTM) as a nitric oxide-dependent modification was re-emphasized. Moreover the authors discussed several techniques, including chemiluminescence-based approaches, the Saville-Griess and 2,3-diaminonaphthalene reactions, immunoblotting methods, and a novel biotin switch that can be used in high throughput fashions to determine subproteomes modified by NO to contain S-nitrosated residues. Other studies

have examined changes in S-nitrosation status of endothelial cell proteins in the setting of vasoregulation and platelet aggregation and explored the role of these modifications within the mitochondria. Such approaches clearly demonstrate how analysis of PTMs when coupled to organelle-based proteomics or proteomics on tissues/cells under different stimulus conditions can yield functional information about the system.

Congenital Heart Disease

Congenital malformations of the heart and circulation not only include examples of most if not all of the other forms of cardiovascular disease but are often characterized by “experiments of nature” that provide opportunities to investigate models beyond the reach of laboratory design. Potential applications of proteomics to congenital heart disease include: 1) hypoxemia, cholesterol metabolism, and the antiatherogenic effects of cyanotic congenital heart disease (61); 2) genetic implications of persistent hypocholesterolemia after cyanotic congenital heart disease is rendered acyanotic by cardiac surgery (61); 3) cholesterol metabolism in rats exposed to controlled experimental hypoxemia (access to tissue rather than plasma); 4) right ventricular myocyte hyperplasia in tetralogy of Fallot and Eisenmenger ventricular septal defect *versus* right ventricular myocyte hypertrophy in primary pulmonary hypertension (71); 5) left ventricular myocyte hyperplasia in congenital aortic valve stenosis *versus* left ventricular myocyte hypertrophy in acquired calcific aortic stenosis (71); 6) essential hypertension *versus* hypertension associated with coarctation of the aorta (65); 7) the induction of myocyte hyperplasia by ischemia in the immature heart *versus* the induction of myocyte necrosis in response to ischemia in the mature heart (71); 8) abnormalities of elastin, smooth muscle, and collagen in the media of dilated great arteries in congenital heart disease (43); and 9) the rare survival of patients to ages far beyond expectations predicted by Gaussian distribution probability (71).

ORGANELLE PROTEOMICS AND CARDIOVASCULAR PHENOTYPES

The many cell types in the cardiovascular system include cardiac myocytes, vascular smooth muscle cells, fibroblasts, and vascular endothelial cells. Although the precise number of proteins hosted by the cardiovascular proteome is debated, the tremendous complexity of the cellular proteomes is widely recognized and is clearly beyond the analytical capabilities of current proteomic technologies. Reduction of this complexity either by dividing the target into more manageable fractions or by focusing on specific organelle proteomes affords an attractive alternative and, more importantly, represents an attainable goal for better spatial and functional correlations of the identified proteins. In this organelle proteomic approach, the identified proteins come with distinct subcellular locations, which are often coupled with the biological processes in which they participate (Fig. 1), thus providing the functional

significance of proteomic data. Among the essential cardiovascular cellular organelles, mitochondria, caveolae, and proteasomes have received attention (Fig. 2)

Mitochondria—A number of investigations have significantly enhanced our understanding of the proteome of mitochondria in mammals. Taylor *et al.* (72) have comprehensively characterized the human heart mitochondrial proteome and compiled a total of 615 distinct protein identifications. However, the majority of proteins were detected by using MALDI-TOF as the identification tool. Mootha *et al.* (73) first reported a repertoire of 186 proteins in mouse heart mitochondria using LC/MS/MS. Although the investigation afforded the first exciting news on cardiac organelle proteome, information was limited regarding the purity and the functionality of the mitochondria used for these studies. In subsequent studies by the same group (74, 75), a much more robust technology platform was used in which 689 proteins were identified from rat liver, heart, and skeletal muscle by using quantitative LC/MS/MS-based proteomic analysis. Novel mitochondrial candidates were further confirmed by protein correlation profiling. Although protein correlation profiling is a novel way to map protein localization, it was confusing to localize proteins from different organelles that had similar sizes or proteins that stick to organelles.

Emili and colleagues (76) first combined proteomic and transcriptomic analysis to characterize protein expression profiles of four organelles (cytosol, membrane-derived microsomes, mitochondria, and nuclei) in six different mouse organs (brain, heart, kidney, liver, lung, and embryonic placenta). 4768 proteins were identified by LC/MS/MS-based shotgun sequencing among which 664 proteins were from murine cardiac mitochondria. The strengths of proteomics for mitochondrial analyses were critically reviewed by McDonald and Van Eyk (77). The authors considered the advantages and limitations of a traditional 2-DE approach in mitochondrial proteomic analysis; alternative methods that have been applied, including different mass spectrometry-based techniques following both one-dimensional gel electrophoresis and gel-free approaches; blue native PAGE; proteome simplification by submitochondrial fractionation; and affinity chromatography. Importantly the combined assessment of protein profiles from various subcellular compartments allowed the observation of intracellular protein translocation, linking proteins into a common context.

Plasma Membrane and Caveolae—The plasma membrane proteome, due to its role as the first interacting surface with other cells and foreign agents, is of particular interest in basic molecular signaling, cell defense, and cell-cell interactions. Furthermore the plasma membrane harbors microdomain invaginations called caveolae, which are known to be highly involved in transport and signaling that regulate diverse cellular functions. These detergent-resistant cholesterol- and glycosphingolipid-coated pits are involved in transcellular movement of molecules and house specialized signaling pro-

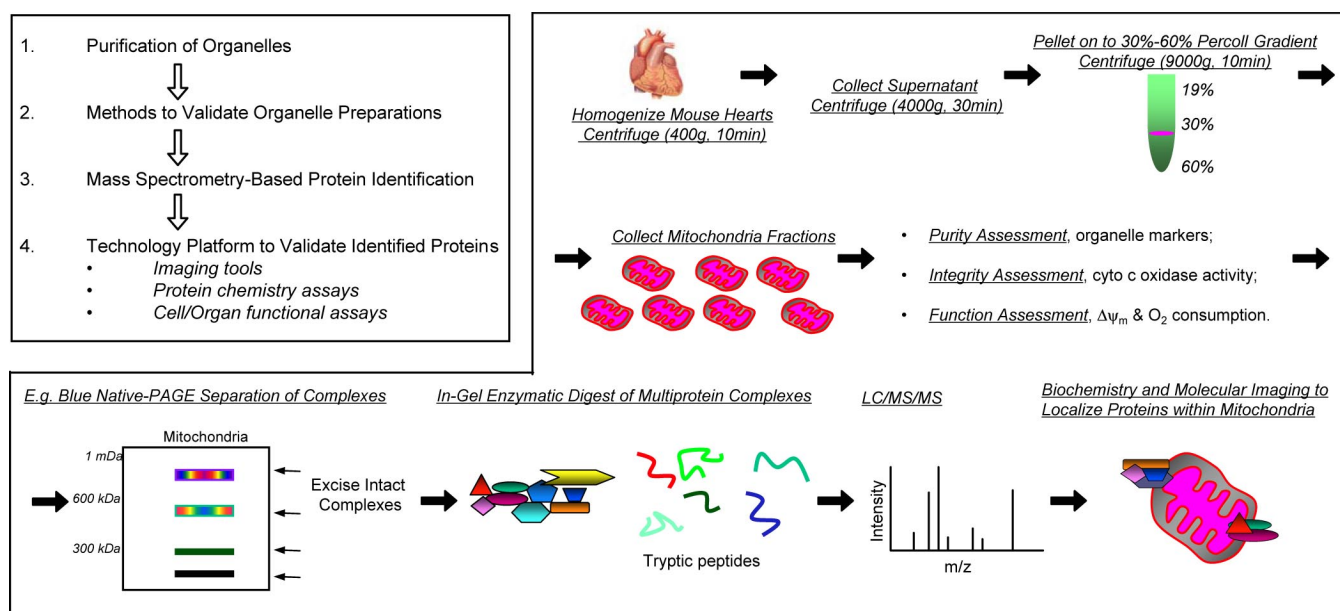


FIG. 1. **General strategy for organelle-based proteomics: example of cardiac mitochondria.** *Top left*, validation of organelle-specific proteins occurs at two independent and complementary steps in the proteomic analysis. First, the functional integrity and anatomical specificity of isolated organelles is evaluated using established biochemical, structural imaging (e.g. electron microscopy), and physiological assays. Second, following mass spectrometry-based protein identification, a battery of techniques are used to confirm the localization of a protein to the given organelle and to investigate its functional relevance therein. *Bottom right*, example of purification and proteomic analysis of cardiac mitochondria. Mouse hearts are homogenized, and a crude mitochondrial fraction is then carried out at three levels: purity (biochemical approaches to rule out contamination from other fractions), integrity (assays to evaluate the intactness of the membranes), and function (studies to determine that the isolated mitochondria behave akin to those in the cell). These mitochondria are then lysed with detergent and analyzed by various separation techniques (shown here is blue native PAGE, a technique that facilitates resolution of intact protein complexes) followed by mass spectrometry-based protein identification of proteins on the basis of LC/MS/MS analysis of tryptic peptides. Lastly the second round of validation for these identified proteins is carried out by localizing them to mitochondria via molecular imaging and evaluating their functional involvement biochemically. *cyto*, cytochrome.

teins, such as endothelial nitric-oxide synthase and caveolin (78). Durr *et al.* (79) examined the plasma membrane from endothelial cells derived directly from rat lung as well as a population of endothelial cells that had been cultured *ex vivo* also from a rat lung source. The investigators used a two-dimensional LC and ion trap tandem mass spectrometry (multidimensional protein identification technology (MudPIT)) to identify 450 proteins from these sample sources. Quite interestingly, many of the proteins (41%) detected from the cells directly isolated from rat lungs were not found in the analysis of the cells that had been cultured beforehand, highlighting the capability of this approach to resolve expression differences between two otherwise similar samples and demonstrating the importance of performing organelle-based proteomics on animal-derived samples (rather than on cell culture models in isolation).

BIOSIGNATURES AND BIOMARKERS OF CARDIOVASCULAR PHENOTYPES

At least three areas of cardiovascular medicine will directly benefit from the development of biosignatures and biomarkers (Fig. 3): first, cardiovascular functional subproteome analyses that serve to delineate mechanistic insights

of cardiovascular pathogenesis; second, cardiovascular or cardiovascular system-related proteomic changes that might be indicative of diseased phenotypes either for the purpose of prognosis or diagnosis; and third, proteomic changes in cardiovascular cells or proteomic changes related to functions of cardiovascular cells that take place over a course of cardiac phenotype manifestation. Potentially these proteomic changes can be induced by treatment with pharmaceutical agents (e.g. β -adrenergic blockade for the treatment of heart failure). The proteomic changes might be used as biosignatures or markers to gain insights regarding the efficacy of a therapy and/or to guide future therapy. This third set of markers, which we designated as “therapeutic markers,” has thus far received limited attention but is highly relevant and clinically important because a significant proportion of cardiovascular diseases are chronic.

Mechanistic Biomarkers—Reactive oxygen species play a critical role in the development of atherosclerosis and have been implicated in promoting potentially pro-atherogenic actions on SMC proliferation, inflammatory cell recruitment, and redox-sensitive gene expression. Because oxidative stress is determined by the balance between pro-oxidants and antioxi-

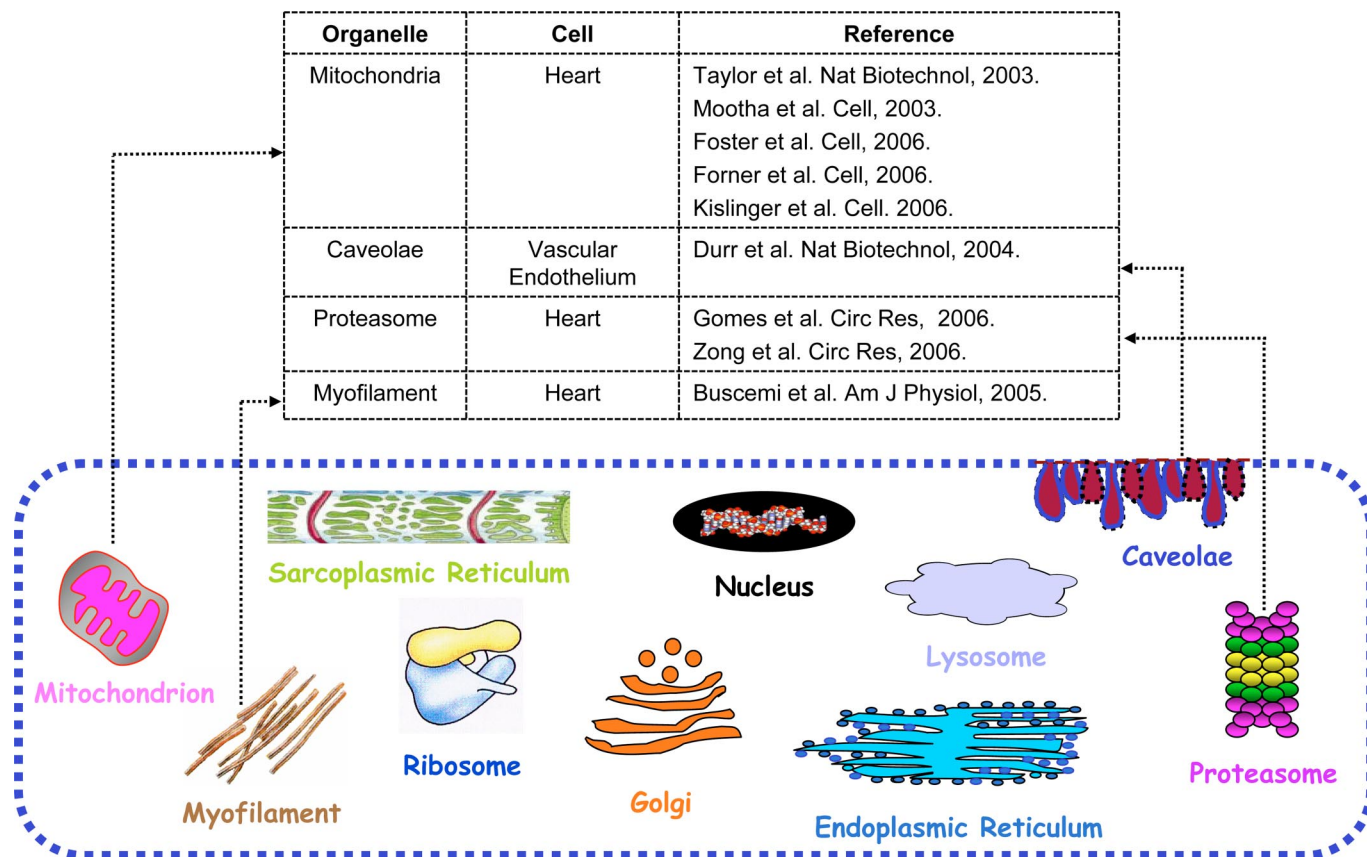


FIG. 2. **Organelle proteomics in the cardiovascular system.** Taylor et al. Nat Biotechnol, 2003, Ref. 72; Mootha et al. Cell, 2003, Ref. 73; Foster et al. Cell, 2006, Ref. 75; Forner et al. Cell, 2006, Ref. 74; Kislinger et al. Cell, 2006, Ref. 76; Durr et al. Nat Biotechnol, 2004, Ref. 79; Buscemi et al. Am J Physiol, 2005, Ref. 3; Gomes et al. Mol Cell Proteomics, 2005, Ref. 96; Zong et al. Mol Cell Proteomics, 2005, Ref. 97.

dants and because alterations in individual reactive oxygen species-generating enzymes are likely to be compensated for by synergistic ones, measurements of individual enzymes at a single time are unlikely to shed much light. A more comprehensive approach may be needed to understand such complex biological systems (80).

We have demonstrated previously that the oxidation status of 1-Cys peroxiredoxin (PRX 6), a novel antioxidant conferring protection against oxidative membrane damage, is a surrogate marker for oxidative stress in cardiovascular tissues (23, 81, 82). The peroxiredoxin family represents a special type of peroxidases because the protein is the reducing substrate itself; upon oxidative stress, the cysteine in the active site is either oxidized to cysteine sulfenic acid or overoxidized to cysteine sulfinic acid. Whereas the first modification is DTT-sensitive and therefore undetectable in 2-DE gels, the latter modification is DTT-resistant and results in a charge shift toward a more acidic pI (83). Thus, peroxiredoxins are encountered as doublet spots, and the ratio of oxidized to reduced protein reflects their oxidation status *in vivo*.

Our proteomic data support the role of oxidative stress in atherogenesis: oxidation of 1-Cys peroxiredoxin was detectable even before lesion formation in young apoE^{-/-} mice and

coincided with a depletion of vascular energy metabolites. Similarly others have shown that overexpression of the uncoupling protein 1 promotes atherosclerosis by triggering mitochondrial dysfunction, depleting energy stores, and increasing superoxide production (84). Thus, there is a growing body of evidence that mitochondrial energy metabolism and oxidative stress are intertwined in atherogenesis. Moreover the oxidation state of 1-Cys peroxiredoxin correlated with lesion size in aortas of old mice, and reduced oxidative stress was associated with successful recovery of the energy pool and alterations of NADPH-generating cytosolic malic enzyme, which provides reducing equivalents for lipid synthesis and glutathione recycling (85). Up-regulation of antioxidant proteins was the last resort rather than the first attempt to confine oxidative stress once other counter-regulatory mechanisms can no longer provide sufficient reducing equivalents to antagonize reactive oxygen species.

These findings were consistent with studies reporting a weak glutathione-related enzymatic antioxidant shield in human atheroma (86). Accordingly proteomics offers a platform to assess simultaneously the expression of multiple pro- and anti-oxidants and to integrate these findings with the oxidation state of redox-sensitive proteins. As such, proteomics

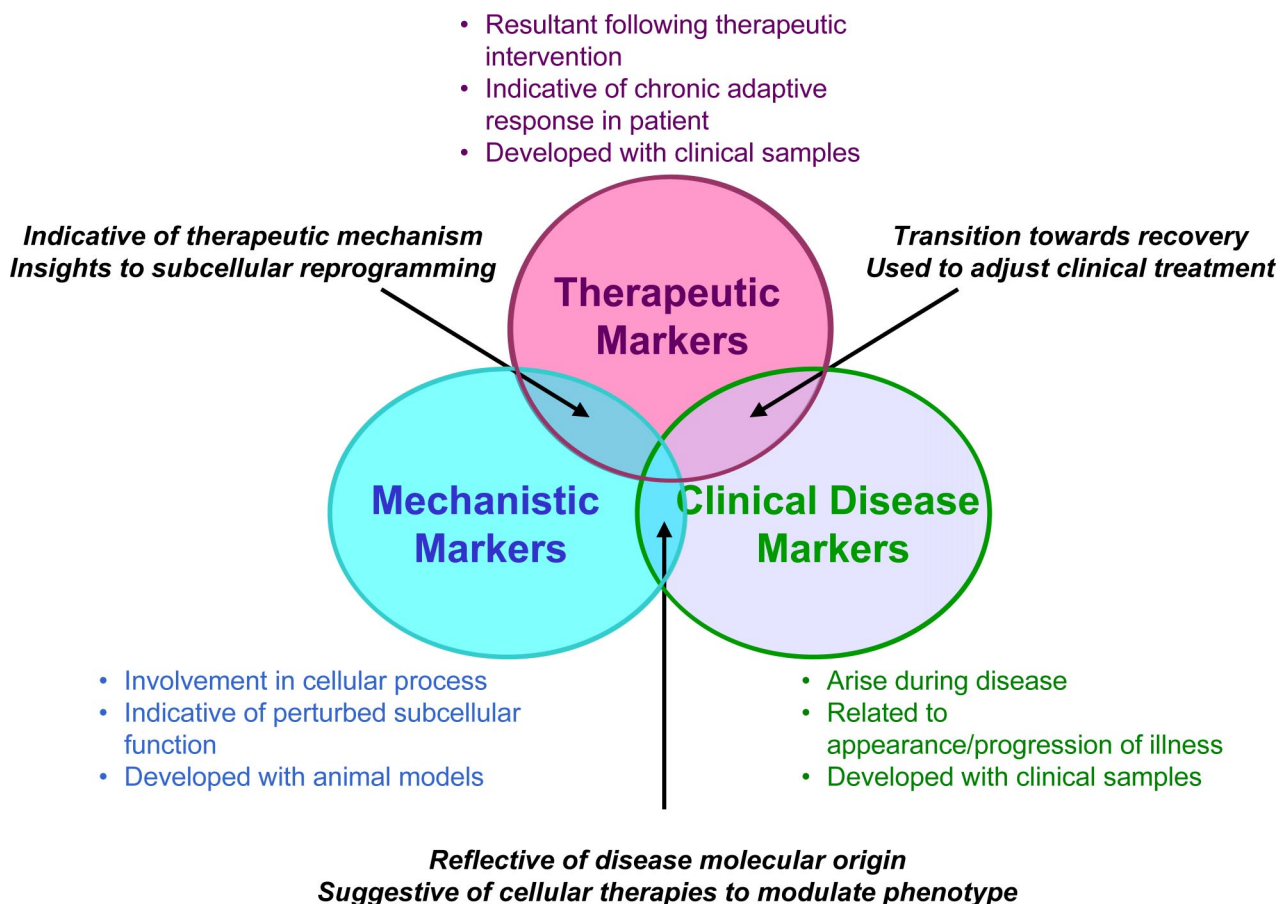


FIG. 3. **Types of biomarkers/biosignatures to be used for cardiovascular disease.** There are at least three different types of biomarkers that can be developed for cardiovascular medicine, including mechanistic markers, clinical disease markers, and therapeutic markers. In the first group, changes in the subcellular phenotype of the organism can lead to alterations in proteins detectable as markers. These changes, constituting mechanistic markers, are closely reflective of what is going on in the cell and how the signaling pathways are manipulated. Second, the arrival of disease carries with it changes in proteins that are detected by proteomics, so-called clinical disease markers. These markers are specific to the individual disease state and can indicate the state of progression, severity, and location of the syndrome. Lastly therapeutic markers are those that become evident as the treatment of a chronic disease progresses in the patient. These markers are influenced by many factors, including individual nature of the disease, drug treatment, patient activities, etc. Under ideal circumstances, a combination of markers from these different groups would be used for a more accurate diagnosis and treatment.

can reveal mechanistic markers of phenotype that are directly indicative of malfunctioning subcellular signaling that results in impaired cell/organ function.

Clinical Biomarkers—Whereas mechanistic markers directly reflect aberrant subcellular processes, clinical markers (*i.e.* markers of the disease state and its progression) may or may not directly reveal insights into altered cellular function. In contrast, these markers are defined by their unique ability to reflect the presence of a disease and the individual characteristics of pathogenesis. Cardiovascular disease in particular has a host of clinical biomarkers that have been in use for many years (*e.g.* troponins). For example, the diagnosis of myocardial infarction has depended on a convincing history, electrocardiogram changes, and the detection of a protein biomarker of myocardial necrosis. These biomarker tests depend on the systemic spillover of either cytosolic (creatinase) or structural, cardiac muscle-specific myofilament

proteins (troponins) because the time course and extent of systemic release correlates well with the extent of cardiac injury. Creatine kinase, however, exists in multiple tissues with appreciable and variable serum activity in the absence of myocardial injury. The advent of more sensitive biomarkers of necrosis such as the MB isoform of creatine kinase and, more importantly, of cardiac muscle-specific troponins has increased biomarker reliability. Troponin T is currently the gold standard for the diagnosis of cardiac ischemia. This change in clinical practice was formalized in consensus recommendations that resulted in a new and already widely accepted definition of myocardial infarction based primarily on the rise (and fall) of highly sensitive and specific biomarkers of myocardial necrosis such as the cardiac troponins (87).

There are also newly emerging protein biomarkers especially for atherosclerosis (88) among which high sensitivity C-reactive protein (CRP) is currently the most promising. The

importance of systemic inflammation in atherogenesis is now beyond doubt, and a plethora of inflammatory proteins, chemokines, and cytokines show an association with early and advanced atherogenesis. Some of these associations, however, do not reflect a “risk factor status” but rather a protective counter-regulatory response to atherosclerosis aimed at limiting inflammation. One of the novel biomarkers for cardiovascular disease is high sensitivity CRP that serves as a pattern recognition molecule in innate immunity that might directly contribute to a proinflammatory state in atheroma by inducing adhesion molecule expression on endothelial cells, stimulating cytokine release of monocytes, and activating the complement cascade (89). Alternatively high levels of CRP may identify subjects capable of producing a prominent inflammatory response to pathogens and other stress factors. This capability has a complex genetic control and was recently shown to enhance the risk of atherosclerosis (90).

Although several studies nicely demonstrate the use of proteomics to promote inductive scientific discovery and to identify mechanistic biomarkers, there are only a few examples to suggest that proteomics is equally successful in delivering new clinical biomarkers. In fact, studies of cancer have already sparked widespread controversy about the use of proteomics in biomarker discovery, and it seems imperative to avoid similar mistakes in cardiovascular research. Cardiovascular medicine has embraced the idea of multiple markers for risk assessment, *i.e.* total cholesterol, high density lipoprotein, low density lipoprotein, body mass index, blood pressure, and family history. However, multi-marker protein models constructed from mass spectrometry peaks of serum proteins bound to chip surfaces may not prove to be acceptable without knowing the molecular entity of these proteins.

There has to be a consensus that the use of expansive and updated technology cannot substitute for validation in biomarker discovery and that it is damaging for the reputation of proteomics if accepted standards for biomarker discovery are not met. Too often validation is based on a small group of patients without adequate controls or proper adjustment for risk factor distribution. Instead high abundance proteins get heralded as “biomarkers” when they might not be justified for “biomarker candidates” because they lack the specificity and the sensitivity required for clinical applications. Importantly for a protein to become a clinical biomarker extensive interlaboratory validation is required, and it is essential to demonstrate that the new biomarkers not only predict disease in different populations but also outperform or improve the predictive value of existing risk factors.

Enthusiasm in the proteomics community was somewhat dampened once the difficulties of biomarker discovery became apparent. Anderson and Anderson (91) made a major contribution by pointing out that plasma proteins span a linear dynamic range of 10–12 orders of magnitude, whereas current proteomic techniques only resolve proteins within 3–4

orders of magnitude (91). Meanwhile there is a noticeable shift in the approach to biomarker discovery. Investigators now deliberately avoid body fluids but instead search for potential biomarkers by comparing diseased and healthy tissues or proximal fluids of the tissue of interest where the potential biomarkers are less dilute and not masked by the 22 most abundant serum proteins that comprise 99% of the plasma protein mass. Whether this will prove to be sufficient to overcome the biological complexity and to deliver the much needed breakthrough for proteomics in clinical biomarker discovery remains to be seen.

Combining Proteomics and Metabolomics—The function of the majority of the genes in most genomes is still unknown, and many proteomic studies are attempting to fill this need. However, because information flows from DNA to RNA to protein to function, the role of each gene product in metabolism also needs to be studied. A recent theoretical study by tk;4ter Kuile and Westerhoff (92) using the methods of metabolic control analysis demonstrated that there is no general quantitative relationship between mRNA levels and function. A comprehensive study of many metabolites together with proteins could be invaluable, and attempts to address this need are being developed, including metabolite target analysis, metabolite profiling, metabolomics, and metabolic fingerprinting

To link alterations of cellular proteins to metabolism and function, we have combined 2D electrophoresis, mass spectrometry, and high resolution NMR spectroscopy with mouse models of cardiovascular disease. Importantly 2D electrophoresis was found to be a suitable technique because it preferentially visualizes soluble high abundance proteins and their post-translational modifications, many of which are key enzymes in glucose, lipid, and energy metabolism. As a first proof-of-principle demonstration, we analyzed mice deficient for protein kinase C δ (93). In murine hearts, loss of PKC δ resulted in altered lipid and glucose metabolism, attenuated reactive oxygen species production, and exaggerated myocardial damage after ischemic preconditioning (81, 82). Similarly PKC δ deficiency interfered with the energy metabolism of vascular SMCs, promoted an antioxidant state reflected by decreased levels of reactive oxygen species and increased glutathione concentrations (82), improved SMC survival, and resulted in accelerated vein graft stenosis. Overall protein and metabolite alterations in the cardiovascular system were remarkably consistent in PKC δ -deficient mice. Similar alterations in glucose metabolism affecting the cellular redox status were found in SMCs and cardiomyocytes under *in vivo* as well as *in vitro* conditions (94). A comprehensive quantitative analysis of proteins and metabolites will help provide a better understanding of such complex biological systems and oblige us to acknowledge the multiple facets of disease pathogenesis.

A “clinical biomarker” (as discussed above) may not only be a gene or a protein but also a metabolite. Metabolic profiles

might be used to predict cardiovascular risk and outcome if the methodology is experimentally robust and reproducible. High resolution NMR currently satisfies all the criteria required for metabolomic studies (95) and is being used to study cardiac biology and physiology both in man and in animal models to obtain information on diagnosis and to monitor therapeutic responses and patient outcomes. Mass spectrometry-based methods offer better sensitivity than NMR-based techniques, but the increase in sensitivity comes at the expense of quantitative accuracy. Coefficients of variations range from 10 to 30% unless a database of metabolites is established and internal metabolite standards are spiked into the samples. Overall mass spectrometry- as well as NMR-based metabolomic techniques have a potential for discovering mechanistic as well as clinical biomarkers and together with proteomic techniques may become an integrated part of future biomarker discovery programs.

CONCLUSIONS

The importance of understanding the mechanisms of cardiovascular disease, both acquired and congenital, reflects the need to reduce human morbidity and mortality. Proteomics is a critical suite of tools designed to address this need from the level of signal transduction, organelle and cellular mechanism, biomarker development, and phenotype analysis. Challenges include biomarker strategies and translation of these strategies to clinical settings. Strong ties between basic and clinical sciences are essential in facilitating this transition. The nature of proteomic analysis on readily available body fluids (blood, plasma, saliva, and urine) has already reduced the hurdles in applying these techniques to patients. As strategies for high throughput quantitation continue to develop, the capability of proteomics to serve as a clinical tool will also increase. The basic science challenge that we currently face is to make sure that the proteomic changes detected in extracted samples are in fact indicative of what is going on *in vivo*. New approaches for proteomics on living cells will help address this need, and further refinement of quantification will ensure that this information has functional relevance to the phenotype under study. Proteomics has emerged as an indispensable method for deciphering cellular mechanisms and for linking these mechanisms to cardiovascular disease and health.

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