

## Transformative Impact of Proteomics on Cardiovascular Health and Disease

### A Scientific Statement From the American Heart Association

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**Abstract**—The year 2014 marked the 20th anniversary of the coining of the term *proteomics*. The purpose of this scientific statement is to summarize advances over this period that have catalyzed our capacity to address the experimental, translational, and clinical implications of proteomics as applied to cardiovascular health and disease and to evaluate the current status of the field. Key successes that have energized the field are delineated; opportunities for proteomics to drive basic science research, facilitate clinical translation, and establish diagnostic and therapeutic healthcare algorithms are discussed; and challenges that remain to be solved before proteomic technologies can be readily translated from scientific discoveries to meaningful advances in cardiovascular care are addressed. Proteomics is the result of disruptive technologies, namely, mass spectrometry and database searching, which drove protein analysis from 1 protein at a time to protein mixture analyses that enable large-scale analysis of proteins and facilitate paradigm shifts in biological concepts that address important clinical questions. Over the past 20 years, the field of proteomics has matured, yet it is still developing rapidly. The scope of this statement will extend beyond the reaches of a typical review article and offer guidance on the use of next-generation proteomics for future scientific discovery in the basic research laboratory and clinical settings. (*Circulation*. 2015;132:852-872. DOI: 10.1161/CIR.0000000000000226.)

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■ proteome ■ systems biology ■ translational research

The term *proteomics* was coined 20 years ago to describe the large-scale evaluation of proteins in a tissue or blood sample, an endeavor with broad applicability to cardiovascular research.<sup>1</sup> Proteomic approaches are used to (1) scan complex biological mixtures and focus on proteins that distinguish

groups; (2) catalogue samples that contain hundreds of proteins; (3) derive structural information from protein sequencing; (4) separate and identify proteins and peptides that differ by posttranslational modification; (5) analyze protein interaction and protein complexes; (6) perform protein quantitation;

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and (7) accomplish genome annotation. In the setting of cardiovascular health and disease, proteomics is useful for identifying pathogenesis and progression of cardiac developmental defects, atherosclerosis, hypertension, myocarditis, cardiomyopathies, myocardial infarction, arrhythmias, heart failure, aneurysms, and stroke.

Proteomic throughput capabilities have increased dramatically over the past decade as a result of technological developments, including advances in sample preparation, mass spectrometry (MS)-based analysis, database searching, and bioinformatics techniques that facilitate data interpretation.<sup>2-4</sup> The translation of proteomic discoveries to meaningful clinical applications in cardiovascular medicine has the primary benefit of providing an unbiased evaluation of complex protein mixtures.

This statement will discuss the current state and our vision for proteomics. It is essential to (1) impart these technologies to current investigators and the next generation of scientists who can benefit from their growing availability; (2) invest in technology to continue reaping the benefits of the proteomic foundations that have been established; and (3) facilitate the technologies to mature further and support them to fruition, similar to project management approaches taken when setting up a business or bringing a new product to market. For the investment to date, proteomic output has been greater than is sometimes perceived but has not yet reached its full potential. We will showcase the strengths of proteomics while defining realistic expectations for this maturing technology. Advances in the proteomics field will also be compared and contrasted to advances in the genomics field to highlight similarities and differences between the 2 approaches.

### The Foundation of Proteomics

The quality and quantity of data provided by cardiovascular proteomics are growing exponentially, in part because of collaborations among proteomic groups initiated by the Human Proteome Organization (<http://www.hupo.org>) and the successful National Institutes of Health (NIH)-sponsored multicenter proteomic contracts. As our ability to detect and characterize alternatively spliced isoforms and post-translational modifications (PTMs) advances, so does our understanding of the complexity of the proteome. Proteome complexity is highlighted by recent studies showing that phenotype is not entirely determined by information encoded in the translated genome; PTMs, noncoding RNAs (including microRNAs), epigenetic changes, and protein-protein interaction networks affect phenotype.<sup>5,6</sup> The ultimate goal of cardiovascular proteomics is to harness and better comprehend this molecular complexity as a means to discover new, effective strategies for the prevention, identification, and treatment of cardiovascular disease.

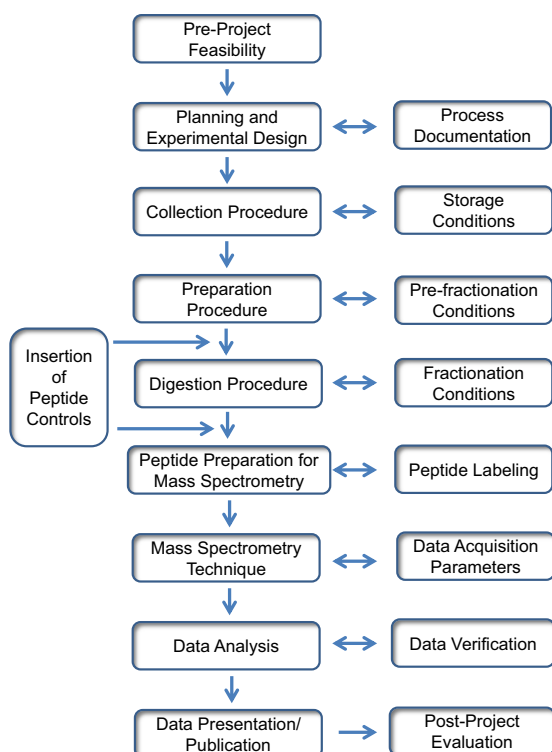
The identification of candidate biomarkers of cardiomyocyte necrosis is a classic example of the use of proteomics to identify novel indicators of cardiac injury.<sup>7</sup> The development of more sensitive troponin assays is another example of how proteomics has enabled the development of improved diagnostic methods,<sup>8</sup> with high-sensitivity troponin assays permitting earlier diagnosis of acute coronary syndromes.<sup>9,10</sup> MS was an integral part of the harmonization of cardiac

troponin I assays, which was accomplished by development of a human cardiac standard to calibrate troponin assays.<sup>11</sup> Previously established troponin assays served as diagnostic tools for patient risk stratification and guided treatment strategies in patients suspected of having an acute coronary syndrome. Increased troponin assay sensitivity has enabled earlier identification and treatment of myocardial infarction, the identification of which was refined on the basis of these assays, which reinforces the significance of proteomic contributions to current clinical practice.<sup>12,13</sup> Other examples of proteomics being used to discover biomarkers for improved diagnostics include the OVADx and OVA1 assays for ovarian cancer.<sup>14</sup> A primary objective of proteomics is improved diagnostic capabilities that increase the ability to separate patients into subgroups, more than the identification of novel “gold standard” biomarkers.

### Protocol Standardization

Over the past 2 decades, considerable efforts have been made to discover disease biomarkers using high-throughput *-omic* approaches. For example, major efforts to identify new clinical markers for cancer and cardiovascular disease have been undertaken; however, the results have been unsatisfactory, in part because of a lack of, or failure to implement, standardized protocols for biomarker discovery. Although numerous discovery workflows for the detection of novel biomarkers have been proposed, many have yielded disappointing results, in part because of a lack of protocol standardization. One issue has been inadequately defined clinical phenotypes of the patient populations from whom samples were obtained. Furthermore, many diseases (eg, coronary artery disease and heart failure) represent a spectrum of conditions in which proteins of interest obtained from samples collected over a period of time may be influenced by treatment, disease severity, and patient behavior. Additionally, the expertise of the researcher at every step from experimental design through sample preparation, familiarity with advanced proteomic equipment, MS analysis, and results integration affect biomarker discovery, and some investigators may not standardize and control each step. Suboptimal protocol standards have resulted in poor reproducibility across laboratories.

For optimal results, proteomics project management details must be considered from initial assessment of feasibility to presentation and publication of results (Figure 1). Although proteomic studies inevitably vary among different investigators, minimal criteria by which to ensure accuracy, reproducibility, and reliability at levels acceptable to the broader scientific community are possible. Criteria for reporting proteomic protein identification data were proposed in 2005,<sup>15,16</sup> and these have been widely adopted in the proteomic research community. Table 1 lists the minimum acceptable proteomic information necessary to validate results for basic, translational, and clinical cardiovascular proteomic studies. Table 1 reflects guidelines established by *Molecular and Cellular Proteomics*, the *Journal of Proteome Research*, and the *Journal of Proteomics* for the publication of proteomic results.<sup>15</sup>



**Figure 1.** Project workflow for a typical proteomics project, with experimental design factors to be considered listed on the left and experimental conditions to be considered listed on the right.

## Experimental Design

A major consideration before one undertakes a proteomic study is the clear definition of its purpose (ie, hypothesis driven versus discovery based). Several major issues must be taken into account in the experimental design of proteomic studies for biomarker discovery, including (1) criteria for subject selection; (2) sample type, collection, and handling; (3) sample storage conditions and duration; (4) proteomic data acquisition technique; (5) data analysis; (6) result documentation; and (7) appropriate strategies for replication of results in independent cohorts.<sup>17,18</sup> Each step in the experimental process requires optimization for the particular type of sample used (eg, tissue, serum, plasma, or other body fluid). The complexity of each step of the process should not be overlooked. When samples are collected over time, for example, variations in patient behavior for the duration of the studies cannot be controlled and must be taken into account. Moreover, many proteomic studies suffer from the lack of proper controls or from use of patient sample sizes that are insufficient to yield a properly powered analysis.

## Lessons From Plasma Proteome Projects

Human plasma and serum are commonly used for clinical and research purposes. For proteomic analysis, sample collection is critical; the choice of anticoagulant agent used for plasma preparations or the lack of an anticoagulant agent for serum collection, as well as the presence or absence of protease inhibitors, affects the activity of proteolytic enzymes in these samples.<sup>19</sup> Although hundreds of serum and plasma proteomic studies have been published, no single optimal anticoagulant agent has been established for these types of samples, in part

**Table 1. Information Required to Maximize Proteomic Experimental Reproducibility**

Main Parameter	Subparameters
Sample preparation	Sample collection, storage, and processing information
Sample digestion	Enzyme(s) used, digestion time
Software for identification and search engines	<ul style="list-style-type: none"> <li>• Name of peak list–generating software (release version and date)</li> <li>• Database searched (release version and date)</li> <li>• Parameters used (default vs altered)</li> <li>• Enzyme specificity considered</li> <li>• Missed cleavages allowed</li> <li>• Number of fixed/variable modifications (residue specificity)</li> <li>• Mass tolerance for precursor ions</li> <li>• Mass tolerance for fragment ions</li> <li>• Species restriction and justification for searching subset</li> <li>• Number of protein database entries searched</li> <li>• Threshold score/E-value for accepting individual spectra</li> <li>• Justification of threshold used</li> <li>• For large data sets, false-positive rate estimation and how calculated</li> <li>• For peptides matching to multiple family members, criteria for selection and how redundancy eliminated/handled</li> </ul>
Software/methods used for quantitation	<ul style="list-style-type: none"> <li>• Quantitation methodology (numbers of peaks, peak intensity, peak area, extracted ion chromatogram)</li> <li>• Minimum thresholds required for data quantitation</li> <li>• Biological and analytical reproducibility addressed</li> <li>• Justification for removal of any outliers</li> <li>• Statistics used to assess accuracy and significance</li> </ul>
Each protein identified	<ul style="list-style-type: none"> <li>• Number of unique peptides identified</li> <li>• Score/E-value for peptide</li> <li>• Sequence coverage identified (%)</li> <li>• Accession number</li> <li>• Precursor <i>m/z</i> and charge</li> <li>• Sequence identified</li> </ul>
Single-site peptide identification	<ul style="list-style-type: none"> <li>• Labeled MS/MS spectrum for each peptide</li> <li>• Protein quantitation measurement and accuracy (mean±SD)</li> </ul>
PTM identification	<ul style="list-style-type: none"> <li>• Software/method used to evaluate site assignment</li> <li>• Labeled MS/MS spectrum included for each PTM</li> </ul>

E-value indicates expectation value; MS/MS, tandem mass spectrometry; *m/z*, mass-to-charge ratio; and PTM, posttranslational modification.

because downstream applications may necessitate the use of different anticoagulant agents (eg, when measuring matrix metalloproteinase activity that is inhibited by EDTA or when assessing PTMs).<sup>20</sup> Some sample preparation protocols induce artifactual modifications, including *S*-thiolation modifications and methylation.<sup>21,22</sup> A report of results collected from 35 different laboratories that analyzed reference samples of human serum, EDTA-anticoagulated plasma, heparin-anticoagulated plasma, and citrate-anticoagulated plasma as part of the Human Plasma Proteome Project showed that EDTA-anticoagulated plasma samples yielded the most reproducible

results, although the range of results was highly variable, largely because of a lack of laboratory selection criteria and a lack of commonly applied analytical standards.<sup>23</sup> These studies and an independent alternative analysis of the data showed considerable diversity in proteins identified in proteomic analysis of samples obtained and processed at different institutions and demonstrated that the compilation of data across multiple experiments was necessary to derive a comprehensive plasma proteome.<sup>24</sup>

Another important issue is the dynamic range of sample complexity. In serum, the concentrations of proteins vary over a wide range, and thus, clinically important proteins present in low abundance may be masked by more abundant proteins. Although removal of high-abundance proteins, in particular albumin, allows lower-abundance proteins to be assessed quantitatively, such removal is challenging and may result in depletion of the analyte of interest if it binds to the albumin fraction, thus affecting reproducibility and quantitative analysis.<sup>25</sup> For certain proteomic investigations, selective analysis of glycoproteins can be used to recover important lower-abundance proteins for evaluation, because albumin is not glycosylated.<sup>26</sup> Other biological fluids that have been evaluated with proteomics include cerebrospinal fluid, saliva, and urine, and these fluids face similar challenges with sample stability, dynamic range, complexity, and reproducibility.<sup>27,28</sup>

To date, >150 cardiovascular disease biomarkers have been proposed.<sup>29</sup> Biomarker validation requires development of reproducible high-throughput assays and analysis of thousands of patient samples to properly evaluate selectivity and specificity in clinical samples.<sup>30</sup> Although MS techniques are available to accurately and reproducibly determine putative biomarkers in a high-throughput manner, specific protocols for each analyte need to be developed. A multiplexed, multiple reaction monitoring (MRM)-based assay of tryptic digests of whole plasma and stable isotope-labeled peptide standards has been used to detect 81 of 135 peptides derived from 67 putative protein biomarkers at attomole levels with excellent linear response ( $r > 0.98$ ).<sup>31</sup> The MRM assay was accomplished in 30 minutes and used ultra-high-performance liquid chromatography (LC) attached to an Agilent 6490 triple-quadrupole mass spectrometer equipped with an ion funnel<sup>31</sup> to increase ion transmission, reduce contamination and chemical noise, and improve overall system signal.<sup>32</sup> MRM studies and mass cytometry are being used as an alternative to flow cytometry approaches, and in the near future, other multilevel platforms could compete with existing analytical measurements, such as enzyme-linked immunosorbent assay tests, in clinical settings.<sup>33,34</sup> Although technical issues presently preclude widespread use of MS for time-sensitive diagnostic evaluations such as ruling out myocardial infarction or stroke, these issues are being addressed.

In order for a biomarker to be optimally clinically relevant, there are several desirable criteria. These include high sensitivity and specificity for the disease it denotes, resistance to degradation, and ease of detection and identification (eg, expressed on the cell surface, secreted, or released from intracellular locations).<sup>18</sup> The 3 stages of biomarker development include discovery, performance evaluation, and impact determination to assess the incremental benefit(s) when added

to current diagnostics.<sup>35</sup> Problems with biomarker discovery often arise more as a consequence of problems with experimental design than because of technological shortfalls in identifying or measuring the biomarker. For instance, tissue biomarker evaluation often does not take into consideration its heterogeneity within diseased tissues, and whole tissue, including both diseased and healthy, is often homogenized for protein extraction and analysis of the combined fraction.

### Lessons From Cancer Biomarker Projects

A single biomarker is unlikely to be suitable for all patients with a particular cancer because of heterogeneity of malignant cells within the patient population. Accordingly, multiple biomarker approaches may prove more advantageous than a single biomarker. The first large panel biomarker screening test for ovarian cancer that uses proteomic techniques, OvaDx, is a microarray-based assay of >100 plasma proteins associated with immune system activation in response to early stage ovarian cancer ([http://www.arrayit.com/Microarray\\_Diagnostics/OvaDx\\_Ovarian\\_Cancer\\_Test/ovadx\\_ovarian\\_cancer\\_test.html](http://www.arrayit.com/Microarray_Diagnostics/OvaDx_Ovarian_Cancer_Test/ovadx_ovarian_cancer_test.html)). OvaDx was approved by the US Food and Drug Administration; its cost-effectiveness is currently being evaluated. This panel-based approach holds promise in the diagnosis of other disease states in which no single biomarker has demonstrated sufficiently robust discriminatory capacity to be clinically useful, such as for acute cerebral ischemia or adverse left ventricular remodeling that progresses to heart failure.<sup>10</sup>

Another test that has been approved by the US Food and Drug Administration that makes use of proteomic results is OVA1 (Vermillion, Austin, TX),<sup>14</sup> a simple blood test for 5 proteins that is used to assess the likelihood that an ovarian mass is malignant.<sup>14,36</sup> In a multicenter case-control study, serum proteomic expressions in women with invasive epithelial ovarian cancer (n=153), nonepithelial ovarian cancers (n=42), benign pelvic masses (n=166), or healthy volunteers (n=142) identified several potential biomarkers for ovarian cancer.<sup>37</sup> These serum proteins were detected with Ciphergen SELDI (Surface-Enhanced Laser Desorption/Ionization)-MS ProteinChip technology (Vermillion).<sup>38</sup>

Although DNA microarrays have permitted global expression studies for nearly all genes, few microarray biomarkers are used clinically.<sup>39</sup> Likewise, proteomics will not uncover new clinical biomarkers until there is suitable rigor in experimental design and analytical capabilities. The determination of change at the level of the proteome, however, has several advantages over determination of transcriptome changes, including detection of functional changes such as PTMs, protein-protein interactions, and protein degradation.

The potential for bias in proteomic biomarker discovery may contribute to low validation rates.<sup>40</sup> Frequently in biomarker discovery studies, control and diseased groups are compared with approximately equal numbers in each sample by use of a Student *t* test for statistical comparison; proteins differentially expressed between the 2 groups are considered as possible biomarkers. In the general population, however, the prevalence of diseased subjects relative to nondiseased subjects is often low. In this circumstance, the identification of a biomarker with the appropriate sensitivity and specificity



requires a larger-scale study with a greater number of control samples. Such studies are best performed with interdisciplinary, multi-institutional, and nationwide or international collaboration. Other potentials for bias include the heavy reliance on mouse models for proteomic studies. In many of these studies, the mice used are young, genetically homogeneous, and without other common comorbidities present in patients, such as diabetes mellitus and smoking. For these reasons, results obtained from mouse studies often do not represent the human population.

### **Membrane and Extracellular Matrix Protein Solubility**

The large-scale isolation and enrichment of membrane and extracellular matrix proteins remains a challenge.<sup>20,41</sup> Protein extraction from cell membranes or the extracellular matrix is difficult because of the hydrophobic nature and poor solubility of these proteins. Poor solubility also limits the utility of 2-dimensional gel electrophoresis for membrane protein analysis. Although multiple approaches to investigate membrane and extracellular matrix proteins have been proposed, limitations persist but are getting addressed.<sup>42,43</sup>

### **Sample Protein Quantitation**

Bradford or Lowry assays are most frequently used to determine protein concentration before sample digestion and MS. Depending on sample preparation methods and the buffer used, however, these assays are not always satisfactory for protein determination, and alternatives such as detergent-compatible methods may be required. Independent validation of protein concentration by 1-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel with silver or Coomassie blue staining to equalize sample loads before comparative analysis significantly reduces intersample variability, thereby increasing confidence in subsequent analysis and interpretation.

### **Protein Digestion**

Digestion of human serum albumin by 12 typical protein digestion protocols revealed that peptide release is nonstoichiometric and involves complex kinetics.<sup>44</sup> This highlights the need for standardized procedures to ensure accurate quantification of protein samples and suitable internal standards and isotope dilution techniques to improve uniformity of proteomic analysis.<sup>44</sup> At a minimum, full documentation of the digestion protocol is required so that other researchers can replicate the results and determine approach utility. A protocol to increase the reproducibility and reliability of proteomic workflows has been proposed and involves the addition of 3 exogenous proteins and 2 sets of isotopically labeled peptides (1 added before tryptic digestion and the other before LC-MS analysis).<sup>45</sup> Digestion protocols for any study should be identical from sample to sample, and precise details should be recorded to evaluate possible variability or bias between different studies. For clinical studies, all samples should be processed in an identical fashion, with sample treatment and storage documented to ensure reproducibility. Although newer robotic platforms may lessen variation, there remains a need for standardization and optimization with each new application or use.

## **Proteomic Technologies**

Advances in proteomic techniques have led to transformative developments in how changes in protein expression and PTMs are detected and quantified. Both technological and methodological advancements allow greater depth of proteome coverage and higher sample throughput. Reduction in sample complexity has been achieved by prefractionation of samples before LC-tandem MS (LC-MS/MS). Prefractionation can be performed by centrifugation, ion exchange, gel filtration, hydrophobic chromatography, or isoelectric separation. Newer prefractionation methods involve techniques to improve sensitivity, such as hydrophilic interaction LC, thereby allowing smaller amounts of sample to be used.<sup>46</sup> Sample complexity is further reduced by the direct coupling of reversed-phase LC columns to MS analysis. Multistage LC separations can also be performed online with MS analysis. Longer analytical columns composed of smaller particle-sized resins (to increase surface area) and greater chromatography gradients improve capacity and resolution of chromatographic separations. LC-MS/MS can also be combined with other techniques such as fluorescence-activated cell sorting to enrich for cell-type-specific proteomes.

Other prefractionation techniques available include 2-dimensional gel electrophoresis, which can resolve hundreds to thousands of proteins in a complex mixture with the added advantage of yielding their isoelectric points and molecular weights. This is particularly useful in the evaluation of substrate enzymatic proteolysis, in which truncated proteins are expected.<sup>47</sup> When combined with MS, 2-dimensional gel electrophoresis can reproducibly identify changes in expression of protein isoforms and PTMs.<sup>48</sup> Improved protein detection and separation protocols have been combined to enhance the resolution of 2-dimensional gel electrophoresis, which provides an example of how improved techniques, and not necessarily instrumentation advances, enhance the results obtained.<sup>49</sup>

Prefractionated or unfractionated samples can be quantified and compared by label-free quantitative proteomics or by labeling specific amino acids in samples (Table 2). Some commonly used proteomic labeling methods include stable isotope labeling with amino acids in cell culture (SILAC), isobaric tags for relative and absolute quantitation (iTRAQ), and tandem mass tags that bind to terminal and side-chain amino groups. Continued improvements in the design and synthesis of isobaric tags now allow up to 10 different samples to be labeled and measured simultaneously. The combination of triplex metabolic labeling and 6-plex isobaric tags permits the simultaneous investigation of 18 samples.<sup>63</sup> The data obtained have allowed investigators to discern temporal abundance profiles for thousands of proteins over a 6-point time course in rapamycin-stimulated yeast.<sup>63</sup> The ability to include both technical and biological replicates that provide the statistical power needed to determine differences among samples while reducing the overall experiment time is advantageous for future proteomic studies.

Antibody-based pulldowns or columns and lectin affinity can be used for enrichment of target proteins (and their noncovalent complexes), with the caveat that the specificity depends on the selectivity of the agent, and the background

**Table 2. Comparison of Proteomic Techniques for Protein Quantification**

Method	Site Modified	Strengths	Limitations
Label-free LC-MS/MS <sup>50,51</sup>	None	<ul style="list-style-type: none"> <li>• Spectral counting</li> <li>• High dynamic range of quantification</li> <li>• Simple biochemical workflows</li> <li>• Whole proteome analysis</li> <li>• Comparison of large numbers of different states</li> <li>• Ion abundance–based measurements</li> </ul>	<ul style="list-style-type: none"> <li>• Each sample has to be analyzed individually (no sample multiplexing)</li> <li>• LC-MS reproducibility, and instrument drift may lead to quantification errors</li> </ul>
SRM <sup>52</sup> ; MRM <sup>53*</sup>	None	<ul style="list-style-type: none"> <li>• Speed</li> <li>• Ease of use</li> <li>• High-quality data</li> <li>• High dynamic range for quantification</li> </ul>	<ul style="list-style-type: none"> <li>• Requires peptide synthesis</li> <li>• Limited to chosen peptides</li> <li>• Bias in peptide chosen may exist</li> </ul>
DIA MS <sup>4,51,54,55</sup>	None	<ul style="list-style-type: none"> <li>• Powerful technique: single sample injection can generate fragment ion spectra for all analytes detectable within a defined precursor range</li> </ul>	<ul style="list-style-type: none"> <li>• Software for analysis of DIA data is limited</li> </ul>
<sup>18</sup> O labeling <sup>56</sup>	Lysine and arginine modification during trypsin digestion	<ul style="list-style-type: none"> <li>• Simple and fast</li> </ul>	<ul style="list-style-type: none"> <li>• Exchange reaction is seldom complete for peptides, which results in a complex isotopic pattern caused by overlap of unlabeled, single-labeled, and double-labeled peptides</li> </ul>
<sup>14</sup> N/ <sup>15</sup> N and/or <sup>12</sup> C/ <sup>13</sup> C labeling <sup>57</sup>	Metabolic labeling of all amino acids	<ul style="list-style-type: none"> <li>• Both partial and full labeling comparable with respect to dynamic range, accuracy, and reproducibility</li> <li>• Both are suitable for quantitative proteomics characterization</li> </ul>	<ul style="list-style-type: none"> <li>• Comparison of only 2 states</li> <li>• Cell culture systems only</li> </ul>
ICPL <sup>58</sup>	Free amino acid	<ul style="list-style-type: none"> <li>• Simple and accurate</li> </ul>	<ul style="list-style-type: none"> <li>• Limited number of samples that can be compared</li> <li>• Isotopic effect of deuterated tags interferes with retention time of the labeled peptides during LC</li> </ul>
SILAC <sup>59</sup>	Metabolic incorporation of arginine or lysine	<ul style="list-style-type: none"> <li>• Best global labeling strategy available</li> </ul>	<ul style="list-style-type: none"> <li>• Expensive</li> <li>• Limited (2–3) number of samples that can be compared</li> <li>• Complex biochemical workflows</li> <li>• Cell culture systems only</li> </ul>
iTRAQ <sup>60</sup>	Peptide N-termini and ε-amino group of lysine	<ul style="list-style-type: none"> <li>• Provides accurate quantification spanning 2 orders of magnitude</li> <li>• Increased MS sensitivity compared with ICAT</li> <li>• Eight samples can be compared at the same time</li> </ul>	<ul style="list-style-type: none"> <li>• Peptide cofragmentation (inadvertently selecting ≥2 closely spaced peptides for MS/MS instead of 1)</li> <li>• Possible contamination of the reporter ion region with the second isotope of the phenylalanine immonium ion on the <i>m/z</i> 121 peak, which can interfere with peptide quantification unless the mass resolution is sufficient to distinguish these isobaric peaks</li> </ul>
TMT <sup>61</sup>	Peptide N-termini and ε-amino group of lysine	<ul style="list-style-type: none"> <li>• Similar quantitative precision and accuracy to iTRAQ</li> <li>• Ten samples can be compared at the same time</li> </ul>	<ul style="list-style-type: none"> <li>• Number of proteins quantified decreases with number of samples compared</li> </ul>
TAILS <sup>62</sup>	Terminal amino group	<ul style="list-style-type: none"> <li>• Can use varying isotopic labeling</li> <li>• Can identify naturally blocked N-termini</li> <li>• Requires relatively small sample amount</li> </ul>	<ul style="list-style-type: none"> <li>• Moderately expensive</li> <li>• Difficult to validate results for single-peptide–based N-terminome analysis</li> </ul>

DIA indicates data-independent acquisition; ICAT, isotope-coded affinity tag; ICPL, isotope-coded protein labeling; iTRAQ, isobaric tags for relative and absolute quantitation; LC, liquid chromatography; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; MS, mass spectrometry; SILAC, stable isotope labeling with amino acids in cell culture; SRM, single reaction monitoring; TAILS, terminal amine isotopic labeling of substrates; and TMT, tandem mass tags.

\*SRM and MRM terms are used interchangeably (because of trademark issues). In most SRM applications, more than 1 precursor to a product pair is monitored; by definition, multiple transitions are monitored in MRM experiments.

may vary from one support to another.<sup>64</sup> Another approach to enrich peptides is through the use of aptamers, unique synthetic molecules selected to bind peptides with high specificity and affinity. An aptamer-based proteomic technique to measure low-abundance proteins in small volumes (15  $\mu$ L) of serum or plasma detected 813 proteins with low limits of detection (1 pmol/L median) and a dynamic range of 7 orders of magnitude.<sup>65</sup> This study identified 60 potential biomarkers of chronic kidney disease, 2 of which were already well established.

### MS Versus Immunoblotting for Targeted Protein Identification

Cell signaling research is based on immunoblotting. Although it is a reliable and powerful approach for detecting an individual protein in a complex mixture, immunoblotting detection is limited only to the predetermined, targeted protein and assumes antibody specificity is sufficient. A major advantage of quantitative proteomics over immunoblotting is that novel cellular processes can be discovered in a nonselective, unbiased manner. Many proteomic laboratories identify proteins and PTM changes by MS/MS and subsequently validate these findings using immunoblotting. However, current MS/MS can be more accurate and reliable than immunoblotting, because many antibodies (especially antibodies to PTMs) show cross-reactivity to nonspecific proteins. Immunoblotting reproducibility is compromised because of the availability of poor-quality antibodies.<sup>66</sup> Immunoblotting as a confirmation or secondary validation of MS identifications should only be required for single-peptide hits or when assignments of multiple peptides is based on low-quality spectra or when the quantitative changes are minute. In either of these circumstances, better MS data can usually be obtained in a subsequent targeted experiment. In situations in which MS can be used, this should be favored over Western blotting, as long as sufficient data are presented to assess the accuracy of the MS results. These data should include accession numbers, number of unique peptides, number of total peptides, mass of peptides, mass of fragments, and search engine scores.

### Proteome Arrays

In addition to ascertaining the presence and relative abundance of various proteins, protein microarrays may also provide information about protein function and potential drug targets.<sup>67</sup> High-throughput proteomics studies have been conducted using functional protein microarrays to study phosphorylation states of the ErbB-receptor kinase family.<sup>68,69</sup> A peptide array that contains 2.1 million overlapping peptides covering all known human proteins was used for the analysis of antibody specificity, which demonstrates the initial feasibility of large protein arrays.<sup>70</sup>

### Next-Generation Proteomics

With the advanced features present in the latest mass spectrometer systems, next-generation proteomics is expected to allow a more in-depth view of the proteome using smaller sample volumes while requiring less time to obtain results.<sup>71,72</sup> One goal in proteomics is to develop platforms that provide

single-cell proteomic data to better understand cell heterogeneity.<sup>73</sup> However, this will require (1) experimental techniques to efficiently fractionate low sample amounts; (2) approaches that enable MS to detect peptides from proteins that occur throughout the protein dynamic range of the cell; (3) exquisite sensitivity to detect subtle signal-to-noise ratios for the lowest abundance peptides and proteins; and (4) an ability to detect and measure protein complexes.

### The Promise of Proteomics

Proteomics holds the promise that information on all expressed proteins can provide a precise map of the current condition of cells, tissues, organs, and whole organisms. A particular strength is that unlike genomic and transcriptomic approaches, proteomics provides important insights into modifications that regulate function and activity of enzymes and other proteins, because it can identify binding sites for specific protein interactions.<sup>74</sup> Abundant examples in the literature are available, including a special issue on cardiovascular disease that focused on clinical and translational proteomics.<sup>75–87</sup> One specific example is activity-based protein profiling with proteomic techniques, which is currently being used to annotate the enzymatic proteome and uses chemical probes that target large groups of enzymes that have similar active-site features.<sup>88</sup> Contributing to the posttranscriptional complexity of the proteome are alternative splicing and isoform variants, often with extensive peptide redundancies, that are not easily addressed by standard rules of parsimony when acquired in the context of multidimensional quantitative proteomic studies. We will present differences between expectations, feasibility, and reality in the use of proteomics as a complete mapping tool by specifically focusing on its use to delineate protein PTMs.

Protein PTMs are well-established regulators of protein activity, and the number of known PTMs is expanding rapidly. In the 2009 release of the Human Protein Reference Database, 93 710 PTM sites were described in 30 047 protein entries.<sup>89,90</sup> An overview across all species in dbPTM 3.0 (accessed November 2014) revealed 221 020 experimentally validated PTMs compared with 14 589 and 36 466 experimentally validated PTMs in version 1.0 (2006) and version 2.0 (2009), respectively.<sup>91</sup> Given the lack of experimental data on the 3-dimensional structure of the vast majority of proteins, it is not surprising that many more putative PTM sites are anticipated from predicted protein structure. The most commonly detected PTMs to date are phosphorylation, ubiquitylation, acetylation, *N*- and *O*-linked glycosylation, and methylation.<sup>91</sup>

Although >200 types of protein PTMs are known, the low stoichiometry and dynamic nature of these modifications complicate their detection and analysis. MS is the best technique to determine PTMs in system-wide approaches. Mass spectrometers are continuously improving, yielding better mass resolution and mass accuracy. Fragmentation technology, an important aspect of mass spectrometers, has also improved with new methods such as electron capture dissociation,<sup>92</sup> electron transfer dissociation,<sup>93</sup> and higher-energy collisional activation,<sup>94</sup> which are complementary to ion-trap collision-induced dissociation. MS that uses electron transfer dissociation allows even labile PTMs to remain intact on the

peptide backbone, thereby facilitating not only PTM detection but also accurate site assignment.

The use of current proteomics techniques to obtain a complete characterization of PTMs remains challenging, for both technical and biochemical reasons previously described in detail.<sup>90</sup> Phosphorylation is the most mature protein PTM field in terms of publication numbers and techniques developed for targeted evaluation. Phosphoproteomics has contributed significantly to our understanding of signal transduction by mapping changes in protein activity associated with phosphorylation status. For example, targeted proteomics of myofilament phosphorylation has increased mechanistic understanding of myocyte contraction, and phosphorylation as a regulator of protein folding and the formation of amyloid species has led to the Alzheimer theory of heart failure.<sup>76,82</sup> Numerous phosphopeptide enrichment strategies exist, including phosphotyrosine immunoprecipitation, immobilized metal affinity chromatography, TiO<sub>2</sub> chromatography, and combinations of these techniques. With the use of phospho-enrichment and MS techniques, 725 high-confidence phosphopeptides were detected in K562 cell lysate.<sup>95</sup> Just 3 years later, 11 995 unique phosphopeptides were detected in human embryonic stem cells.<sup>96</sup> Another recent example is the description of temporal dynamics of thrombin signaling in endothelial cells, which identified and analyzed a total of 2224 sites subject to thrombin-mediated phosphorylation.<sup>97</sup> Such deep mapping can support development of novel inhibitors of the coagulation cascade. Another similar study is the characterization of downstream effects of mammalian target of rapamycin.<sup>98</sup> This pathway controls phosphorylation of 335 proteins including carbamoyl-phosphate synthetase 2, which stimulates de novo synthesis of pyrimidines and controls cell proliferation. Proteomic studies into other PTMs, such as modification of proteins by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc), are also evolving. In a recent study, diabetes mellitus-associated hyperglycemia led to increased *O*-GlcNAcylation of AKT at the T430/T479 amino acids, which through further downstream cascades, induces vascular calcification.<sup>99</sup> Although glycosylation is a widespread PTM, its analysis is challenging because of its labile nature. The enrichment of *N*-linked glycoproteins in human serum has been accomplished by inclusion of a small *N*-glycan chip (40 nL volume) to an online nano-LC and high-resolution time-of-flight (TOF)-MS.<sup>100</sup> A combination of different MS fragmentation methods had been used for analysis of *N*-glycosylation on proteins secreted from endothelial cells. Identification of important PTMs associated with cardiovascular disease has also improved with high-throughput glycosylation profiling, acetylation and *O*-GlcNAc protein modifications.<sup>101–103</sup> Acetylation and methylation are among the next PTMs for which improved methods are being developed; although these modifications are themselves stable, high resolution and high mass accuracy are necessary to separate or distinguish acetylation ( $\Delta=42.010565$  atomic mass units) from trimethylation ( $\Delta=42.046950$  atomic mass units). Notwithstanding these technical advances, system-wide analyses are hindered by the lack of advanced analysis software that can accommodate the dynamic changes of multiple PTMs on a single protein.<sup>71</sup> Fortunately, progress is being

made in this direction, specifically with respect to multisite discrimination of histone PTM combinatorial complexity.<sup>104</sup>

Beyond studies of individual PTMs, advances have been made in the study of interactions between PTMs. Baek et al<sup>105</sup> described downregulated phosphorylation of the brain sodium channel Nav1.2 after kainite-induced seizures with concomitant upregulated methylation at adjacent sites, which suggested reciprocal regulation of these 2 PTMs. In the specific context of histones, Garske and colleagues<sup>104</sup> used a MALDI-TOF (matrix-assisted laser desorption/ionization TOF) MS approach to demonstrate novel chromatin phosphorylation and methylation sites that interact with each other and mediate binding of chromatin binding proteins such as histone H3. Taken together, these examples illustrate that proteomics has evolved beyond description of protein expression and can provide insights into regulation of protein function that is critical for the development of new therapies.

### Discovery Proteomics Versus Targeted Protein Evaluation With MS

Both discovery proteomics and targeted protein evaluations have their benefits.<sup>81,83,106–109</sup> In discovery proteomics, strategies include bottom-up and top-down approaches.<sup>106</sup> Bottom-up strategies (peptide-level identification) are based on analysis of peptide fragments derived from parent proteins. In contrast, top-down proteomic analysis (protein-level identification) allows the identification of intact proteins and is the only approach that can potentially determine the full set of PTMs that are present on a single protein molecule, thereby allowing investigation of the interrelationships of substoichiometric PTMs.<sup>108,110</sup> This approach has recently been successfully used in the assessment of the phosphorylation pattern of proteins in acutely infarcted swine myocardium, where 3 target proteins (cardiac troponin I, myosin regulatory light chain, and enigma homolog isoform 2) and their phosphorylation sites were identified.<sup>107</sup> These recent findings complement an earlier report on phosphorylation of troponin I in heart failure and are in keeping with the concept of a crucial role of cardiac protein PTMs in the pathophysiology of cardiovascular disease and a potential role of biomarkers of disease.<sup>76,81,82,109</sup> Once proteins have been identified, several approaches can be taken for targeted proteomics to focus on measuring proteins of interest. Targeted strategies include selected reaction monitoring (SRM), MRM, cyTOF mass cytometry, data-independent acquisition MS that is also a discovery method, aptamers, and *N*-linked glycan chips (Table 2). Open-source software that helps users build SRM methods is available to focus on biological questions.<sup>111</sup> There is the potential for development of thousands of SRM assays for human, mouse, and yeast proteins, and many SRMs have been validated and are available via new online resource sites such as the SRM Atlas (<http://www.srmatlas.org/>).

MRM has been used in other fields, such as chemistry, for >5 decades but has only in the past few years been widely used in proteomics. Data-independent acquisition MS, also known as sequential windowed acquisition of all theoretical (SWATH) MS, is the acquisition of MS data on a fast, high-resolution quadrupole-quadrupole TOF instrument with repeated cycling through, for example, 32 consecutive 25-Da precursor



isolation windows to facilitate identification of peptides over a dynamic range of 4 orders of magnitude with consistency and accuracy comparable to SRM.<sup>54,112</sup> Full-mass-range data-independent acquisition has been used by several laboratories for more than a decade.<sup>4,51,55</sup> The use of affinity purification coupled with data-independent acquisition MS resulted in the reproducible quantification of 1967 proteins across different stimulation time points, which provided important insights into the 14-3-3 $\beta$  interactome and the dynamic changes that occur after insulin growth factor-1 stimulation.<sup>113</sup> One limitation of targeted MS is that one can only measure what is already known. Unknown proteins, or unknown modifications, are missed, and a modification that moves sites within the same peptide sequence (eg, phosphorylation-shifting sites) can confound data interpretation. Altogether, this combination of unbiased, broad-range precursor ion fragmentation and targeted data extraction reduces many of the limitations of current proteomic methods and is a powerful technique that will continue to be used in the future.

Targeted proteomics can be used to obtain more precise, quantitative, and sensitive results.<sup>114</sup> One common approach for targeted proteomics is to use affinity targeting methods.<sup>115</sup> The recovery and abundance measurement of peptides that contain a particular PTM, such as tyrosine phosphorylation or ubiquitylation, can be increased substantially by enrichment for proteins with this particular modification. This can be achieved by use of affinity resins or antibodies that specifically select for signature sequences or modifications. Dual-selection methods that combine 2 affinity strategies can further increase the number of PTMs detected.

### The Human Proteome

The Human Proteome Project, a broad-based international effort spearheaded by the Human Proteome Organization, aims to detect all proteins predicted by the Human Genome data, relate them to human health and disease, and provide high-quality reference data and analytical resources available to the community.<sup>116</sup> Very recently, 2 independent groups of investigators published their initial efforts toward obtaining the complete proteome of specific human tissues or individual cell types.<sup>117,118</sup> Using high-resolution Fourier-transform MS, a draft map of the human proteome was obtained from 17 adult tissues, 7 fetal tissues, and 6 purified primary hematopoietic cells.<sup>117</sup> Altogether, the proteins identified were encoded by 17294 genes (84% of the total annotated protein-coding genes in humans). In another investigation using public MS data as well as in-house MS data, a human proteome database called ProteomicDB was developed.<sup>118</sup> This repository covers 97% of 13378 genes with annotated evidence at the protein level and 84% of 5531 genes with evidence at the transcript level.

Mapping a human plasma proteome has advantages for clinical translation in the context of biomarker research but disadvantages for studies into disease-specific pathways where analysis of affected tissues provides better signal-to-noise ratio than plasma. In certain contexts, proteomic progress is perceived as slow and results as inconsistent across laboratories. For example, proteomic approaches have failed to realize their full potential in the diagnosis of acute stroke. Although acute stroke biomarkers have been identified,<sup>119</sup>

few studies have attempted to validate their clinical utility or define the clinical context in which they would be used.<sup>120</sup> Another unmet clinical need is the development of sensitive and specific biomarkers for predicting preeclampsia. With traditional biomarkers not generally fulfilling clinical needs, the condition has been the subject of a number of proteomic studies, the results of which are inconsistent and only marginally overlapping.<sup>121–123</sup> These shortcomings, however, are not attributable to proteomic techniques but to the suboptimal design of studies, limited sample size, substandard sample collection, and lack of replication in independent cohorts. Thus, to fully realize the potential of proteomic approaches to improve patient care, unmet expectations can be overcome by adhering to rigorous standards that apply to other types of biomarker studies.<sup>124</sup>

A promising strategy is the study of cellular and subcellular proteomes, particularly in circumstances in which cells can be obtained by a minimally invasive method and are available in sufficient quantities to facilitate proteomic analysis. From a cardiovascular point of view, blood cells are of interest for the study of key pathophysiological principles, including inflammation, cell adhesion, and coagulation. For example, a recent study comprehensively mapped the platelet proteome, taking advantage of highly purified washed platelet samples.<sup>125</sup> In keeping with previous studies, the authors describe  $\approx$ 4000 individual proteins, with roughly 80% of the platelet proteome remaining stable within and between donors. On the basis of these data, the next steps include comparison of patients with genetic or other clinical conditions affecting platelet function, studies into PTMs, and investigation of the effects of age, sex, and medications on the platelet proteome.<sup>126,127</sup> Subproteome evaluations provide insight into subcellular distribution of proteins and have also been undertaken successfully for platelets.<sup>128</sup>

Proteomics is most effective when combined with other technologies or approaches rather than used as a stand-alone method. Integration of multiple *-omics* data can reveal differentially regulated networks at transcriptomic and proteomic levels.<sup>125,129</sup> Technological advances that have improved sensitivity, accuracy, and throughput of sample analysis have resulted in increased volumes of data with greater complexity in downstream processing and interpretation. Consequently, advances in bioinformatics are important to the successful application of proteomics, in terms of dealing with a voluminous quantity of information, increasing magnitude of proteome complexity (eg, considering the combinatorial possibilities of all PTMs), and maximizing abstraction of meaningful interpretation.

### Systems Proteomics

Proteomic technologies and experimental strategies continue to advance and widen the scope and reach of biological insight in cardiovascular medicine. The resulting expansion in data set magnitude and complexity mandates robust analytical approaches. Effective democratization of both data analysis and interpretation remains a key limitation for proteomics. Although proteomic repositories and databases of protein information (such as UniProt, <http://www.uniprot.org/>) are making admirable attempts to accomplish this, the data are

still heavily processed by the time they are shared with subsequent investigators. Currently, raw data files are very large, and current methods of processing are labyrinthine and in many cases unmodifiable because of the use of proprietary software. These limitations prevent straightforward data sharing. Inclusive systems strategies to synthesize and interrogate high-throughput information have emerged to maximize data interpretation.<sup>130,131</sup> In this regard, complex network analysis provides the capacity to compile, integrate, and extract biological insight from proteomic and multilevel *-omics* studies<sup>132</sup> while obviating requirements for selective data removal. Applied in combination with complementary systems profiling elements (ie, ontological categorization, statistical enrichment and overrepresentation analysis, pathway analysis, and modeling), network templates provide a judicious, comprehensive means of prioritization and prognostication based on extant biological knowledge, thereby identifying context-dependent candidates and pathways for functional validation and iterative refinement (Figure 2).<sup>130,131,133–137</sup>

### Data Analysis, Interpretation, and Sharing

Preliminary data analysis tools have been widely used for protein quantification, such as MaxQuant (<http://www.maxquant.org/>) and OpenMS (<http://open-ms.sourceforge.net/>). In addition, for tandem data analysis, different approaches have been taken, such as de novo sequencing software for identifying previously unknown proteins/peptides by use of PEAKS (<http://www.bioinformatics.com/>) and search tools using existing protein databases, including Mascot (<http://www.matrix-science.com/>), SEQUEST (<http://fields.scripps.edu/sequest/>), and X!Tandem (<http://www.thegpm.org/>). Output from these packages includes both absolute and relative peptide and protein quantification, which require further interpretation for complete functional analysis.

Sharing of meta-data has been beneficial for the validation of data generated by groups from different parts of the world, as well as for improving knowledge about different disease states.<sup>138</sup> Highly regarded proteomic repositories include the Proteomics Identification Database (PRIDE), PeptideAtlas, Proteomics DB, and Global Proteome Machine (GPM). These databases are not yet as well organized or integrated as genome databases such as the Cancer Genome Atlas (TCGA) or the International Cancer Genome Consortium (ICGC), which currently contains >10,000 cancer genomes. The need for better integration is important in facilitating novel future analyses that may not be evident at the time of compilation. As with genomics data, a major limitation is neither technical expertise of the instrumentation nor cost of the instrument or processing but rather ease of data access and analysis. A future focus of attention will be the development of methods to harness acquired results to better understand mechanisms of cardiovascular disease.

### Ontological Composition and Statistical Enrichment Analysis

Ontology assessment enables a summary overview of acquired proteomic data, either for individual proteins before network generation or after assembly into a collective network neighborhood. In its simplest form, the allocation to each protein of

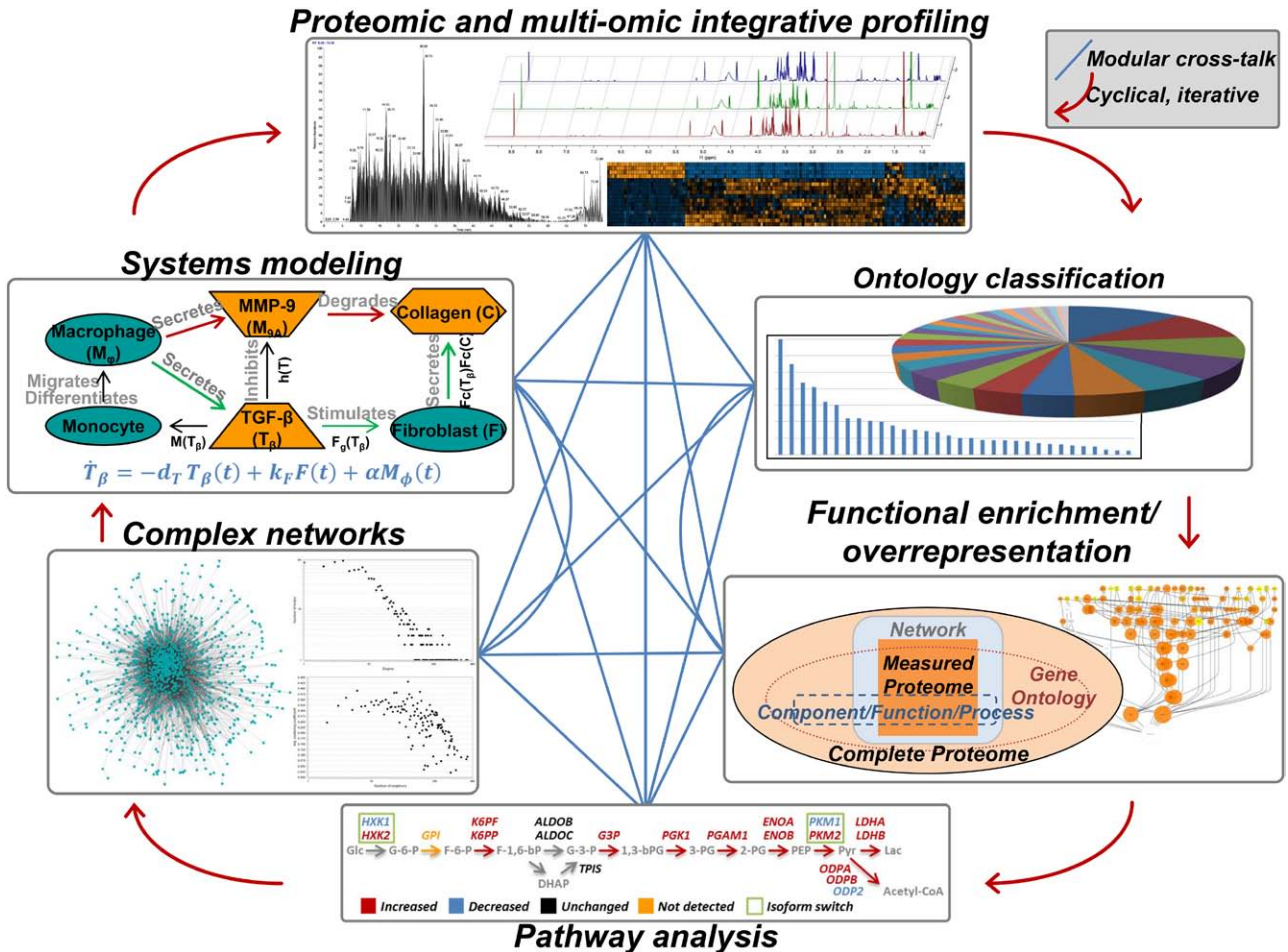
a single Gene Ontology term or alternative platform-restricted classification promotes data reduction and comprehension by clustering a large number of proteins into a reduced set of specified functions.<sup>139–141</sup> Many proteins, however, harbor multiple Gene Ontology associations, which precludes solitary assignment. To address functional multiplicity, representation of individual biological processes, molecular functions, and cellular components is more appropriately addressed by data set overrepresentation or statistical enrichment relative to an established background such as the full proteome (Figure 2).<sup>139–146</sup> Stand-alone options for calculating overrepresentation and functional enrichment are often incorporated into pathway and network analysis algorithms.

### Pathway Analysis

Pathway analysis extends functional categorization through collective documentation of proteome annotations, functions, and interactions in the context of established pathways and current knowledge. Numerous resources exist for pathway interpretation, including protein-protein interactions, metabolic pathways, signaling pathways, pathway diagrams, transcription factor and gene regulatory networks, protein-compound interactions, genetic interaction networks, and protein sequence databases.<sup>131</sup> Importantly, a complementary resource designed specifically for systems-level analysis in cardiovascular proteomics was introduced recently.<sup>147</sup> Using these repositories, proteomic data can be assessed for extent of categorical representation or statistical enrichment within particular signaling and metabolic pathways, or from Gene Ontology-specific or other algorithm-specific functions, processes, and cellular components (Figure 2).<sup>143,144,148</sup> In more expansive commercial applications, functionality extends to disease- and development-associated functions<sup>140,143</sup> and to upstream analysis for identification of predictive causative agents or cellular processes responsible for observed effects. Furthermore, these particular resources facilitate generation of biologically oriented networks.<sup>139,140,143,149</sup> Collectively, these features make pathway analysis a vital data interrogation and hypothesis-generating component of systems analysis pipelines.

### Complex Network Analysis

Contextualizing data within networks, proteins of interest as nodes/vertices are connected through established structural, functional, and regulatory relationships as edges/links (Figure 2). Network generation provides a focal point for collective interpretation of often disparate information<sup>130,131,139,140,143,149</sup> and can also introduce functionally relevant, hypothesis-generating targets overlooked by initial proteomic measurements.<sup>149</sup> Nonstochastic connectivity imparts topological properties, such as network robustness or positionally important proteins such as hubs and bridging nodes, which enables prioritization of potential targets for subsequent investigation.<sup>149,150</sup> Network templates also facilitate multiplatform integration of proteomics with complementary high-throughput data, such as transcriptomes, microRNAs, and metabolomes. Multiple-level integration offers a means to resolve synergistic, emergent behavioral properties that are not directly evident by examination of individual data platforms



**Figure 2.** Systems proteomics as a modular, cyclical, and iterative pipeline. Systems interpretation is facilitated by extension of proteomic and integrative high-throughput profiling from mapping and quantification of protein identities, abundances, and posttranslational modifications to ontological categorization, functional enrichment and overrepresentation assessment, pathway analysis, complex network generation and interrogation, and mathematical modeling, all of which are facilitated by repositories of accumulated knowledge and their accessibility. Systems proteomics can be applied linearly, as a cyclical, iterative process (red arrows, clockwise from top) or as a network of interchangeable modules that may be included and ordered as required (central core of blue lines). Cyclical iteration conveys the concept that systems analysis may lead to actionable hypotheses amenable to further experimental assessment for predictive or model validation. Modules may also be included in multiple instances in a single study, particularly the ontology and overrepresentation modules, which can be performed on raw data, during pathway analysis, and on generated networks. This is in part depicted within the statistical enrichment and overrepresentation module, in which proteomic data (orange rectangle) can be assessed separately or as part of a network (solid blue rectangle) for ontological components, functions, or processes (dashed blue rectangle), determined relative to a reference background such as the complete proteome (large ellipse), of which only a portion with defined Gene Ontology associations (dotted red ellipse) is represented. Output may be viewed in graphical form as in ontology classification or as nested hierarchical networks as shown, here generated with the Cytoscape module BiNGO. Network analysis facilitates integration of multiple sources of high-throughput data on a single template, which yields topological parameters (adjacent graphs) that provide foci and targets for hypothesis generation and experimental validation, as well as a template for computational and mathematical systems modeling, prediction, and simulation. Modeling is dependent on enzyme and binding kinetics determined either empirically or with enzyme databases, in addition to prior information regarding protein-protein interactions and pathway-specific configurations, and is enhanced by the extent of quantitative information available, such as relative or absolute protein abundance, preferably acquired over multiple temporal measurements. Specific requirements will vary, however, depending on the modeling system applied, as outlined in the text. Pathway analysis proteomics data are modified from analysis of the glycolytic pathway described in Folmes et al,<sup>135</sup> whereas systems modeling data are modified from Ghasemi et al<sup>136</sup> and Jin et al.<sup>137</sup> MMP-9 indicates matrix metalloproteinase-9; TGF- $\beta$ , transforming growth factor- $\beta$ .

in isolation. Thus, the compilation of proteomic data by collective biological interactions rather than as simple lists provides distinct advantages for interpretation, interrogation, and actionable prognostication, which makes complex network analysis a valuable component of systems proteomics.

**Network Modeling**

The incorporation of dynamics into network analysis is in its infancy; however, longitudinal analysis such as time series or

phenotype progression offers a powerful tool for disease prediction, diagnosis, and monitoring of therapeutic outcome.<sup>151</sup> As a consequence, an emerging component of systems proteomics is the application of spatiotemporal modeling, including recent forays into the cardiovascular proteomic field.<sup>137,152</sup> Ordinary differential equations, partial differential equations, and stochastic differential equations have been applied to model temporal and spatial changes of biological and physical variables in continuous format.<sup>133,134,153</sup> Partial differential



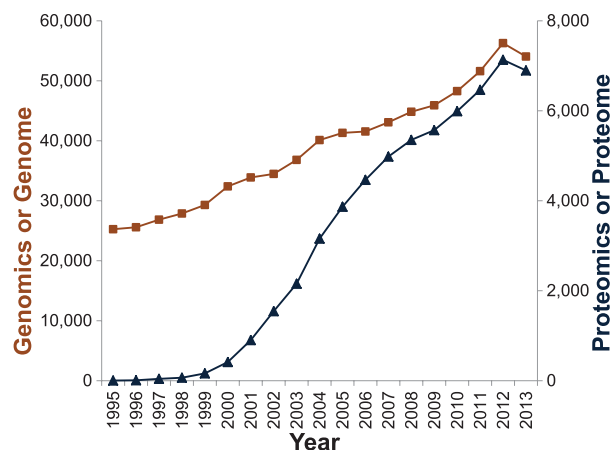
equations have been applied to characterize spatial changes of biological systems, and stochastic differential equations are used to model biological processes when dynamics are partly driven by noise. The most common modeling method uses ordinary differential equations, which are able to infer the temporal, nonlinear dynamic nature of high-dimensional regulatory mechanisms and predict the behavior of a system based on the constructed model (Figure 2).<sup>133,134</sup> All differential equation models require parameters that represent interaction strength or kinetic rates of molecule binding. Kinetic rates of reactions in ordinary differential equation models can be determined from enzyme databases such as BRENDA ([www.brenda-enzymes.org](http://www.brenda-enzymes.org)) and SABIO-RK ([sabio.villa-bosch.de](http://sabio.villa-bosch.de)) or by incorporating experimental results. Interaction strength can be determined by correlation analysis or estimation. Beyond differential equation models in continuous format, there also exist Boolean network models, network ontology analysis, and switching state space models for the nonstationary nature of the network.<sup>151,152,154–156</sup> These modeling methods generally require prior information of associated regulatory mechanisms, including protein-protein interactions or configuration of specific pathways. Available analysis methods also integrate structural properties of networks to classify proteins into functional groups.<sup>152,157,158</sup> Proteomics data with accurate protein quantification facilitate the examination of regulatory interactions among proteins such that every regulation may be considered as an input and output relationship. Although these regulatory processes may not be controlled by direct molecular interactions, modeling can shed light on hidden indirect associations among proteins.

The systems proteomics tools described here form a small fraction of those currently in use by the broader systems biology community. Concerted efforts are required to enable biologists and medical researchers to master these network and systems-level approaches. The effectiveness and success of systems proteomics teams require the inclusion of individuals with computational and mathematical biology expertise.

### Academic and Translational Performance Metrics

Metrics of success are useful for demonstrating value to the scientific community and communicating when goals are making a broader impact. To date, major metrics used for proteomics projects include peer-reviewed publications (research productivity), number of project grants using these technologies (research cross-fertilization), number of trainees interested in proteomics research projects (future research sustainability), and number of projects that translate to new therapies or commercialization (translational research).

A search of the terms *proteomics* or *proteome* shows that since 1994, publications have increased rapidly, with >6000 each year for the previous 3 years (Figure 3). In contrast, the terms *genomics* and *genome* have appeared in publications since 1943 and been used 15 times more frequently than *proteomics* or *proteome*. Publications from the National Heart, Lung, and Blood Institute (NHLBI) Proteomic Centers are cited  $\approx 8.2$  times (mean number of citations), with a mean journal impact factor of 8.0, which is  $\approx 7\%$  greater than the average



**Figure 3.** Annual frequency, beginning with 1995, of publications using the terms *genomics* or *genome* (in orange) compared with *proteomics* or *proteome* (in blue). Note that the scale for the proteomics y-axis is  $\approx 7.5$ -fold higher than for the proteomics y-axis.

NIH investigator. These statistics reveal that the NHLBI-funded proteomics centers have served as strong center points for cardiovascular proteomics research.

Table 3 offers a comparison of genomics and proteomics developments over the past 25 years, to highlight the similarities in forward progress. Although the timeline in Table 3 begins in the 1990s, technological improvements started in the 1970s, when Sanger et al<sup>161</sup> sequenced the first genome, and continued into the 1980s with the first protein identifications by MS. Table 3 is not meant to be a direct comparison but rather reinforces the notion that both genomics and proteomics have made great strides in the past decade. Our understanding of the genome has evolved to include deep RNA sequencing and chromatin immunoprecipitation (ChIP)-sequencing approaches to evaluate DNA accessibility. Similar evolutions are occurring in the proteomics field, including the recent drafts of the human proteome.<sup>117,118</sup>

A search of the NIH Reporter revealed that from 2007 to 2011,  $\approx 1600$  projects involving proteomics were supported across the NIH, with funding for the majority provided through traditional R01 awards. Roughly 60% focus on applied research and 30% on technology development. One index not easily quantified is the number of grant submissions that contain proteomics as a dedicated section of the application. This would allow us to capture a sense of the interest from the general research community, as well as the success rate of grants using proteomics in relation to average success rates.

An important index of the impact of proteomics is the number of junior investigators applying proteomic techniques in their research. Currently, the NIH funds >560 training grants that contain the keyword *proteomics*, including K, F, T, and R15 grants, with funding spread over 21 administering institutes and centers (with the National Institute of Diabetes and Digestive and Kidney Diseases funding 45% and the NHLBI funding 10%), for a total funding of >\$184 million to junior investigators. Although these numbers cannot capture how many more applied for funding, it does indicate widespread incorporation of proteomics at the training



**Table 3. Comparison of Genomics and Proteomics Developments Over the Past 25 Years**

	Genomics	Proteomics
Complexity	Human genome: ≈19000 Genes	Human proteome: ≈2 Million proteins (estimated, reported range 1.8 to >10 million) <sup>159,160</sup>
Improvements in technologies with time	1990 → 2010: Entire genome can be mapped	1990: 200–500 distinct protein spots resolved in 2DE gels 2010: Better MS techniques and better spectral databases result in thousands of protein identifications per sample
Throughput and cost	1 genome: 1990 10 y/\$1 billion 2014 1.5 y/\$1000	96 2DE gel spots analyzed with 6 h of digestion time: >30 h/\$10 000 14 h/<\$2000 (with new gel-free protocols, 2DE gels are less used, and proteome-wide analysis is closer to being possible)
Publications using	Genomics or genome:	Proteomics or proteome:
1995	25 256	3
2014	45 439 (1.8-fold increase)	7618 (2539.3-fold increase)
Experimentally validated PTMs	Not applicable	2006: 14 589 2009: 36 466 2014: 221 020
Patents as of May 2014	1 230 000	634 000

2DE indicates 2-dimensional gel electrophoresis; MS, mass spectrometry; and PTM, posttranslational modification.

level. Of note, these projects are not fully supported by the funding levels provided; the costs of mass spectrometers and other equipment items and supplies are greater than award amounts. Therefore, training grants must be coupled with larger equipment grants and center awards to their mentors, as well as by institutional support for service contracts. Although many investigators already use proteomic approaches in their research, this knowledge also needs to be available to the wider scientific training community. Table 4 shows options for training programs, with different tiers targeting students, fellows, and faculty.

A search of Google patents (accessed June 2014) revealed 634 000 patents filed for proteomic technologies compared with 1 230 000 for genomics and 21 300 for metabolomics. Patents ranged from methods that improve accuracy of detection or quantitation (eg, labeling or fractionation strategies) to diagnostic indicators for particular pathologies and models of functional proteomic simulation scenarios and other bioinformatics tools. Although the success of individual patents is highly variable, the fact that there were >500 000 reveals a burgeoning maturity of proteomics applications.

A final metric to consider is the ultimate translation to commercially available biomarker assays that may provide novel and accurate ways to screen for disease, dictate individualized therapies, or predict response to surgical interventions or medical therapies. For example, clinical information is combined with cholesterol assays to predict cardiovascular

**Table 4. Options for Proteomics Research Training**

Commitment*	Venue	Target Audience	Topics Covered
1–6 h	Online webinars and training videos	Students to established investigators	Sample preparation, how to interpret mass spectrometry results, and basic informatics tools; the goal of these short courses would be to familiarize researchers with individual proteomic approaches
2 wk to 1 semester	Off-site or on-site courses	Students to established investigators	Those listed above, combined into a structured course
1–3 y	Individual laboratories or research teams	Students and postdoctoral fellows	Those listed above and hands-on training for analysis of samples from start to finish for 1 or multiple projects

\*Costs for training provided by academic laboratories or professional societies are much more modest than the tuition for courses offered by commercial vendors.

risk and, as such, is incorporated into clinical guidelines.<sup>162</sup> Although grounded in clinical data, there is room for advancement in cardiovascular disease prediction or management by use of proteomics-based biomarker approaches. Such strategies have the potential to improve health status and clinical effectiveness by directing aggressive strategies to those at highest risk while avoiding expensive or invasive treatments for individuals identified as being at low risk. The pipeline for assay development requires small-scale laboratory-based studies, validation on well-defined clinical cohorts, and robust validation in a large population. From a technical perspective, a putative marker might be identified and tested in a laboratory setting by use of highly accurate but lower-throughput approaches (eg, 2-dimensional LC MS/MS), then further developed by targeted but only moderately high-throughput assays, such as MRM. At this point, biomarker(s) validated in a small population might be moved to industry; however, this would likely involve a small biotechnology or start-up company developed through collaboration with an academic institution that has appropriate safeguards in place to avoid or manage conflicts of interest. The end point for a predictive marker in modest-sized validation cohorts would be investment and development by a larger diagnostics company and US Food and Drug Administration approval after testing in a large population. The rigor of assays developed in small biotechnology companies and moved toward regulatory approval falls into the tier 1 category in a recent best practices consensus by a group of experts in the field, which included many people from the NHLBI Proteomics Centers.<sup>163</sup>

### Emerging Conduits for Science and Applications

Specific recommendations for future proteomics research include the following priorities: (1) Develop methods that accelerate the process of translation and retrotranslation. (2) Manage the process of translation. Not many identified

proteins will be stand-alone biomarkers. A context-specific biomarker is more likely to be feasible for translation to the clinic than a biomarker identified by random sampling methods. (3) Improve communication to the public and research communities to help develop accurate expectations and perceptions of the process of translation. (4) Continue resource allocation to allow maximum potential for transformation. It is crucial to capitalize on advances already made and foster continued investment that will bring these objectives to fruition. A list of future directions in cardiovascular proteomics is provided in Table 5.

Future directions for proteomics should include strategies to ensure transformative activities and applications. For >10 years, the NHLBI has supported proteomic technology development and the application of these technologies to gain greater biological understanding of clinical cardiovascular diseases. This effort has had far-reaching effects, not only on the immediate proteomics community but also on the broader cardiovascular research community. This is reflected in the increased number and scope of proteomic approaches used to address mechanistic questions by the cardiovascular research community and in the maturation of applied technologies for high-throughput proteomic studies. To fully leverage these and other proteomic approaches for biological insight and facilitation of translational research, an integrated and systematic approach involving academia, industry, and funding agencies is imperative.

Past NHLBI Proteomics Center awards have generated tremendous outreach within the cardiovascular research community; cross-fertilization with other NIH institutes (eg, National Cancer Institute and National Institute of Diabetes and Digestive and Kidney Diseases) would likely provide additional synergism. Consideration should also be given for systemic reorganization to facilitate translational activities across industry, government, and academia, such as the U54 Clinical and Translational Science Awards funding mechanism. The importance of maintaining large-center contracts is that they provide extensive resources that translate into biological insights that cannot be accomplished from individual laboratories.

Strategies to stimulate reverse translation from clinical observations to the bench and to better merge clinical and basic science projects to facilitate translation are also needed. After potential targets are validated, high-throughput methods to show protein function are beneficial. In addition to protein concentrations, phenotype information is required to provide a more complete analysis, which entails prespecifying the minimum amount of clinical information that needs to be captured to ensure adequate phenotype assessment. Databases should be established that can store data from across the span of resources (eg, cell-based models, rodent models, large animal models, zebrafish and *Caenorhabditis elegans* comparative biology assessments, computational biology resources, and clinical trial results).

Implementation of additional high-throughput applications for proteomics is also essential. For example, to accomplish pharmacoproteomic approaches for predicting individual patient response to drug therapy, a systems proteomic approach needs to be implemented that incorporates response heterogeneity to stratify different subtypes of patients, together with

**Table 5. Cardiovascular Proteomics: Future Directions**

Technology development—Continue to improve:

1. Deep proteomics coverage of tissue and cell proteomes
2. Proteomic throughput, scale, reliability (eg, establishment of appropriate standards)
3. Methods to assess protein/protein or protein/nonprotein interactions, PTMs, activity-based profiling, protein localization
4. Integration of different data types (eg, physiology, mass spectrometry, histology, NMR imaging) into 1 database platform to better assess interactions and develop computational models
5. Methods to work with dynamic data and take spatial and temporal effects into consideration
6. Data accessibility to the bioinformatician and tools availability to the biologist and clinician; methods to foster cross talk across disciplines

Biological/mechanistic insight—Use proteomics to:

1. Understand response of body, tissue, tissue region, or cell through continuum process in disease and aging (eg, left ventricle; extracellular matrix, mitochondrial, or nuclear fraction; myocyte, endothelial cell, fibroblast, vascular smooth muscle cell, or leukocyte proteomes during response to myocardial infarction)
2. Establish high-throughput assays that accelerate validation/exclusion decisions for targets (eg, cell based activity assays to assess protein function)
3. Identify consequences of selectively blocking or activating target(s) at systems biology level
4. Develop panels of markers that better predict outcomes
5. Establish methods to harness individual variability of single cell, tissue, or organ to develop models of response
6. Develop tools that promote understanding across disciplines and allow teams of biologists, clinicians, and pharma to advance targets

Clinical utilization—Use proteomics to:

1. Determine signature of chronic diseases (eg, atherosclerosis and heart failure) and acute diseases (eg, myocardial infarction and stroke); identify subsets of patients within a disease by first focusing on extreme responders
2. Develop point-of-care technologies for diagnosis and monitoring of treatment efficacy for both acute and chronic treatment strategies (eg, short- and long-term outcomes for patients with congenital heart disease or hypertension)
3. Prioritize biomarker trials for those with actionable drug targets
4. Triangulate proteomics, genomics, and clinical data to improve diagnostic potential with the goal of confident detection at early stages of disease
5. Assess human cardiovascular diseases that lack good animal models
6. Prioritize diseases with largest clinical gaps (eg, heart failure with preserved ejection fraction)

Technology development includes mass spectrometry and bioinformatics research needs; biological/mechanistic insight includes basic science and early translational research needs; and clinical utilization includes clinical trials and diagnostic or prognostic needs.

NMR indicates nuclear magnetic resonance; and PTMs, posttranslational modifications.

identification of a minimum panel of markers necessary to discriminate among various strata. In turn, this information could be applied to model and explore mechanisms as a means of predicting outcomes at the individual level. Successful modeling that coincides with human phenotypic response would then inform and guide cycling back from the bench to clinical implementation.

It is a challenge to be both broad in scope and large in sample capacity. Thus, we need ways to maintain scale-up proteomics while also retaining the ability to divide and restructure experimental processes. One approach is to voxelate extremely large projects: divide into pieces, thoroughly examine individual components, and then reassemble to reconstruct the large picture. This may be achieved within individual research groups or by project division into separate laboratories, each with a degree of specialization required for their particular input.

Mass spectrometers should be more accessible to provide proteomics and biomarker research on a global scale and thereby increase availability to biologists at large. Although spectral analysis will continue to be the forte of the spectrometrist, biologists should be equipped to perform targeted proteomics by establishing interfaces that obviate technical complexity. Technological advances, such as miniaturization, will continue to increase MS accessibility for biology laboratories. Although advances in MS technology are important to the mass spectrometrist from a technical perspective, they are particularly useful to a biologist or clinician with regard to results. Consequently, attempts to democratize proteomics are needed. Genomics has effectively accomplished this by bringing capabilities, such as gene arrays, into routine use by most laboratories, whereas newer, cutting-edge technology-based approaches such as next-generation sequencing remain the purview of specialized genomics laboratories. Although this is a natural by-product of maturation in a field, proteomics can borrow from this template by taking advantage of miniaturization and hardware and software simplification while simultaneously developing keys for proteomics maps and tools for use by diverse groups.

A major limitation to wider application in more laboratories and in clinical research is the cost of equipment, maintenance, and personnel involved in running state-of-the-art MS instruments. To fully realize the potential of proteomics,

a cohort of experts need to be enlisted who can bridge the technological expertise required with an understanding of the pertinent biological questions. Unfortunately, many proteomic cores are run by staff with expertise in instrumentation but little skill in educating biologists regarding experimental designs to make optimal use of the appropriate technology. On the other hand, the most sophisticated equipment cannot be used to its full advantage by biologists or other scientists who do not possess a working understanding of its basic principles or the inherent information content of the data. It is thus beneficial to develop miniaturized, economical, and robust instruments that could be operated by nonspecialized laboratories for general proteomics applications.

### Conclusions

In conclusion, the proteomics field has evolved dramatically over the past 20 years, with advancements and improvements in experimental designs, sample preparation protocols, MS (both equipment and approaches), and analysis, all of which have resulted in substantial forward progress towards a proteomic pipeline to establish cause-and-effect mechanisms of cardiovascular disease. The proteomics evolution has included a better understanding of the need to consider cardiovascular proteomics that resolve protein topology to accelerate membrane protein analysis, identify protein-protein interactions, and elucidate signaling networks in play over the spatial and time continuums of cardiovascular disease processes. The NHLBI has invested significant resources for proteomics centers, which have established transformative technologies that are driving basic biological investigations and propelling clinical translation. The necessary tools are being assembled, and enhancement of communication across disciplines will help to manage implementation and reduce the time required for larger-scale projects. In summary, this is an exciting time for cardiovascular proteomics research.

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\*Modest.

†Significant.



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\*Significant.

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