Clinical proteomics involves the analysis of protein expression of disease proteomes, with the aim of solving a specific clinical problem. Discoveries made from proteomic-based studies contribute to the growing need for innovative medical diagnostics for disease detection. Taking into consideration the global health burden of cardiac disease, clinical proteomics is a valuable tool to improve risk stratification associated with this disease. In cardiovascular medicine, the identification of novel proteins, or biomarkers, that are differentially expressed in cardiac disease proteomes may enable early detection of the disease state, thereby preventing progression to disease end points. This review outlines various proteomic platforms and their technical advancements and relates these to the cardiovascular sciences.

The entire protein complement of the cell, or proteome, is dynamic and changes in response to the disease state. Proteomic-based experiments can be used to characterize such alterations in protein expression during disease progression. With combined improvements in mass spectrometry (MS) technology as well as innovative molecular biology screening tools, there has been widespread growth in the characterization of cardiac disease proteomes. In fact, proteomic technology has been an important tool in the analysis of heart failure (HF), cardiac hypertrophy, and dilated cardiomyopathy. Several of the overarching aims of such proteomic studies remain the identification of maximal proteins and protein complexes, and the precise mapping of posttranslational protein modifications. Traditionally, gel-based approaches have been used to uncover abnormal protein expression patterns with considerable success. However, some traditional gel-based approaches, such as 2D gel electrophoresis (2DE), tend to suffer from limitations largely related to comprehensive protein identifications, which can be partially addressed using gel-free techniques. With the development of increasingly powerful instruments capable of increasingly accurate analysis and greatly improved separation methods and experimental design, the field of proteomics continues to grow, and innovations in research and development will augment previous proteomic profiling experiments.

Proteomic Technologies and Methodologies
Although MS of small molecules has been used for >50 years, with a wide variety of ionization techniques (eg, electron ionizations, fast atom bombardment, chemical ionization), these ionizations were not readily applicable to large molecules, such as protein or peptides. The introduction of mild ionization technologies capable of ionizing large, intact biomolecules such as protein or peptides has allowed MS-based proteomics to develop into a mainstream application.

Matrix-assisted laser desorption/ionization (MALDI) was originally developed by Hillenkamp and Karas, and is based on sample molecules in an absorbing matrix being bombarded with a laser that results in the sample becoming ionized. Tanaka and Saito further improved the concept by mixing fine metal particles with the glycerol liquid matrix, which protects the analyte from the harm of the laser used for
ionization. Electrospray ionization (ESI), invented by Fenn and colleagues in the mid-1980s, is similar to MALDI, results in the mild ionization of large biomolecules. In contrast to MALDI, analyte molecules are dissolved in the liquid phase and ionized by the application of a high voltage (2–4 kV) directly to the solvent, resulting in a fine aerosol at the tip of a column. This ionization is ideally suited for coupling with high-resolution separation technologies, such as liquid chromatography (LC) or capillary electrophoresis, and is currently the most commonly used ionization in MS-based proteomics.

MS-based technology has become more sophisticated in its ability to identify and quantify low-abundance proteins not previously detectable. This ability has largely been aided by the use of recent quantitative methods. For instance, ICATs (isotope-coded affinity tags) were developed with the goal of allowing for improved quantification and analysis of low-abundance proteins. In this technique, cysteinyl residues are labeled using the ICAT reagent, which contains stable isotopes in conjunction with a linker portion that enables incorporation of the isotope itself. A similar approach is SILAC (stable-isotope labeling by amino acids in cell culture) developed by Ong et al. Here, nonradioactive, isotopically labeled amino acids (ie, stable isotopes), namely deuterated leucine residues, are incorporated into mammalian cell lines during the growth phase. Most recently, 13C forms have been used as heavy isotopes to improve chromatographic coelution (ie, mainly incorporated into lysine and arginine). This technique has the added benefits of having near-complete incorporation of stable isotopes. Originally developed exclusively for the quantification of cells grown in culture, this procedure has recently been implicated for the relative quantification of tissue proteomes by metabolically labeling entire model organisms.

Three years after the development of SILAC technology, Schmidt et al introduced ICPL (isotope-coded protein label) in an effort to improve quantification of proteins that were not detectable in proteomic experiments previously, such as proteins found in tissue extracts, bodily fluids, and cellular membrane. The inherent advantage of ICPL lies in the fact that stable isotope tags are incorporated on frequent amino acid residues of isolated proteins (ie, lysine).

The basic tenets of isotopic labeling have also been used in the innovation of iTRAQ (isobaric tags for relative and absolute quantitation), a method that can multiplex as many as 8 samples simultaneously. This technique involves covalent labeling of the N-terminus of peptides with different isobaric tags with reporter ions. Peptides are then identified on the basis of their unique tandem MS (MS/MS) signatures, which enable accurate identification of peptides that may even be in low abundance. This proteomic approach has been used successfully in the analysis of numerous tissues and cells.

Generally, there are 2 different strategies for the MS-based analysis of proteins. One of the more applicable strategies for the analysis of complex proteomes is called bottom-up proteomics. This strategy is the most commonly used proteomic workflow and is applicable to virtually every commercially available mass spectrometer. In this strategy, proteins or complex proteomes are digested to smaller peptides using sequence-specific enzymes, such as trypsin or endoprotease Lys-C. The resulting peptides are separated by LC and analyzed by ESI-MS. Here, separated peptides are ionized at the source (ESI), and the mass to charge (m/z) ratio of peptides is determined in the MS scan (ie, parent/precursor ion mass). Peptide ions are selected for fragmentation by the mass spectrometer in a process called data-dependent MS/MS, using collision-induced fragmentation through collision with inert gas molecules (eg, helium). Alternative fragmentation mechanisms, such as electron transfer dissociation and electron capture dissociation, are also available and have become more popular in recent years. The resulting MS/MS spectra contain valuable information regarding the peptide amino acid sequence of the fragmented ion.

Alternatively, proteins could be analyzed by top-down proteomics. In top-down proteomics, intact proteins are directly ionized and fragmented by either electron capture dissociation or electron transfer dissociation, and the resulting fragmentation pattern is used for protein identification using de novo sequencing approaches. This strategy is currently less applicable to complex protein mixtures and, therefore, is mainly used for the analysis of purified and enriched proteins, although significant improvements have been made in recent years. One of the main advantages of top-down proteomics is that very high sequence coverage can be obtained for the analyzed proteins. This in turn largely eliminates the peptide-to-protein inference problem (ie, ambiguity in identifying a protein from sequenced peptides) that enables the identification of protein isoforms. The accurate assignment of post-translational protein modification is another advantage of top-down proteomics. The group of Kelleher pioneered the use of top-down proteomics in recent years.

### Gel-Based Separation

Gel-based approaches have played a central role in the elucidation of novel markers of cardiac disease. In fact, the majority of proteomic-based studies in muscle continue to rely on gel-based methods in which 2DE is used to separate a complex protein sample. Following separation, proteins are excised from the gel, and corresponding peptide sequences may be analyzed by MALDI-time-of-flight MS, a sensitive technique applied to various complex tissue samples. Here, a nanosecond laser pulse is used to desorb peptides from a matrix cocystal; the resulting ionized biomolecules are accelerated through a flight tube and resolved according to their time of flight, which can be converted to obtain their molecular mass. This technology has recently been applied to improve the diagnostic potential of an existing clinical biomarker. Increases in B-type natriuretic peptide have been correlated with increased risk of cardiovascular events and development of HF and is currently used as a clinical diagnostic tool. This has been noted that natriuretic peptides have some limitations in the diagnosis of HF, such as significant biological variability and dependence on noncardiac influences, such as β-blocker use, body mass index, renal function, and age.

In patients with HF, recent developments have used 2DE to identify leucine-rich α-2-glycoprotein overexpression in cor-
onary sinus serum as a stronger and earlier indicator of HF than B-type natriuretic peptide and determined that its detection is independent of interference from external contributing factors. Two-dimensional gel electrophoresis has been used not only to identify biomarkers, but also to characterize the binding and function of previously uncharacterized cardiac proteins. For example, evidence has suggested a potential role for phospholemman in the modulation of sodium/potassium exchange activity. Crude ventricular tissue was subjected to 2DE taken from phospholemman knockout mice and showed a significant increase in Na-K-ATPase activity through analysis of protein expression levels. Gel electrophoresis has also been recognized for its ability to monitor changes in protein expression levels during disease progression, thereby offering unique insight into the involvement of such proteins in the disease phenotype. Buscemi et al analyzed temporal protein expression patterns in cytoplasmic and myofilament fractions in Rac1 transgenic mice, a model of dilated cardiomyopathy. Proteomic profiling allowed for the identification of tubulin α and β chains, malate dehydrogenase, and manganese superoxide with significant alterations in expression in transgenic mice. Interestingly, these proteins fluctuated in a temporal manner from day 7 to 9, suggesting a role in establishing disease phenotype.

Inherent to 2DE is the ability to detect some posttranslational modifications at the protein level, which may occur during disease progression. Posttranslational modifications are responsible for some of the key biological changes in the function and regulation of proteins. These modifications may be revealed by slight, albeit significant, shifts in the isoelectric point or molecular weight of a single protein (see Van Eyk for a recent review). For instance, Chu et al showed that in phospholamban knockout mice, they could monitor ~3300 protein spots on 2D gels and quantify ~666 of these spots. In that study, increases in expression of myosin light chains was determined as expected as well as an increase in expression and a slight shift in the location of the spot corresponding to acyl-coenzyme A dehydrogenase indicative of a posttranslational modification that had occurred in the phospholamban knockout heart, which was suggested to contribute to the alterations in energy use in this disease state. More recently, García et al carried out 2D gel-based proteomics from human atrial appendage tissue for patients undergoing heart surgery for atrial fibrillation versus controls with sinus rhythm. Twenty-two spots were differentially expressed between the 2 patient groups, with 15 proteins being differentially regulated. Several of the differences were due to posttranslational modification of proteins and not a net change in expression.

Additionally, gel-based separation techniques have been used in conjunction with LC-MS. Yin et al applied this approach recently in their proteomic analysis of myofilament subproteomes. Cardiomyocytes were stimulated with isoproterenol, a β-adrenergic receptor agonist known to activate phosphorylation of many myofilament proteins. Tryptic in-gel digestion of bands of interest from the complex myofilament fraction yielded peptides that were subsequently separated by LC using a reverse-phase column. Resultant peptides were subjected to analysis using an LTQ-Orbitrap mass spectrometer (Thermo Scientific). This combination of a gel-based approach with LC-MS identified low-abundance kinases and phosphatases that may play an important role in the regulation and activation of cardiac contractility.

Gel-based methods have contributed to our understanding of disease development. In addition to the ability to separate thousands of proteins and produce visual data, there are also some limitations that should be carefully considered. For instance, 2D gel-based techniques are biased toward the detection of highly abundant proteins and are limited in the detection of hydrophobic proteins, those with extreme isolectric points, and molecular weights. Moreover, membrane proteins are usually underrepresented because of their poor solubility in the isoelectric-focusing sample buffer and resolution in 2DE. Additionally, although significant progress has been made in 2D gel methods, the lack of automation of extraction, difficulty in digestion, and the required analysis of each spot continue to make 2DE labor intensive. The recent use of computer-controlled image analysis software packages and fluorescence-based staining protocols (ie, differential in gel electrophoresis) has significantly improved 2DE. Separated protein spots are excised, in-gel digested, and eventually identified by MS. Alternatively, proteins can be separated by molecular mass using 1D gel electrophoresis (1D SDS-PAGE), but the entire gel is cut into individual blocks followed by in-gel digestion and resulting peptides are extracted from the gel. This workflow, called GeLC-MS (gel-enhanced LC-MS), is routinely used in modern proteomics laboratories.

Taking Out the Gel

In an effort to address some of the challenges faced in 2DE, several gel-free methods have been developed that enable in-depth identification of proteins in cardiac proteomes, with a significant improvement in detection depth (ie, number of identified proteins). The development of a gel-free approach first subjects complex protein samples to enzyme digestion, such as with trypsin or endoprotease Lys-C, after which peptides are separated using microcapillary LC followed by ESI-MS. Initially, peptides were separated by single-dimension reverse-phase LC. Washburn et al and Link et al subsequently improved the chromatographic resolution of separated peptides using online 2D chromatography, an approach termed MudPIT (multidimensional protein identification technology). Here, peptides are separated first by strong cation exchange chromatography, thereby separating on the basis of charge, and then by elution through a reverse-phase resin, resolving peptides on the basis of hydrophobicity. This approach could be done online where chromatography columns are directly in line with the mass spectrometer, or the peptides/proteins could be separated offline, collected, and subsequently analyzed by 1D reverse-phase LC-MS. Eluted peptides are then electrosprayed into a mass spectrometer through ESI. Following fragmentation, resultant MS/MS spectra are recorded and searched against relevant protein databases, using one of several available spectral matching algorithms, thereby providing a snapshot of large-scale protein expression at a given point in time (Figure).
To maximize protein coverage with the capabilities of sophisticated mass spectrometers, a prefractionation step often is performed to minimize sample complexity. Typically, tissues are first homogenized and then followed by differential centrifugation, providing multiple subcellular fractions that can be analyzed individually. This critical step can increase protein coverage significantly. Alternatively, various other fractionation methodologies based on chromatography or microdissection could be used as well. The end, simplifying input information could be used as well. In the end, simplifying input methodologies based on chromatography or microdissection could be used as well. In the end, simplifying input methodologies based on chromatography or microdissection could be used as well. In the end, simplifying input methodologies based on chromatography or microdissection could be used as well. In the end, simplifying input methodologies based on chromatography or microdissection could be used as well. In the end, simplifying input methodologies based on chromatography or microdissection could be used as well.

We have used the MudPIT approach in the study of a mouse model in which ventricular tissue was extracted from mice with dilated cardiomyopathy, resulting from an Arg to Cys mutation in the phospholamban gene PLN-R9C. We identified 6190 proteins, of which 593 were revealed to be differentially expressed between wild-type and R9C hearts. More recently, we examined an activated calcineurin transgenic mouse model of cardiac hypertrophy and identified and quantified 1918 proteins, 290 of which were differentially expressed between wild-type and hypertrophic hearts. Analysis of the differentially expressed proteins revealed an increase of endoplasmic reticulum stress proteins but limited apoptosis, suggesting that the overexpression of activated calcineurin offered protection against apoptosis, and further analysis of the proteomic data determined α crystallin-B as the downstream modulator of cardiomyocyte protection from apoptosis in activated calcineurin mice.

Even before the onset of proteomic study, the mode by which samples are obtained and handled is an important consideration. For instance, to study the impact of preanalytical variables on outcomes, plasma samples obtained from healthy samples anticoagulated with EDTA, citrate, or heparin were studied. Subsequently, clustering of m/z data corresponding to each sample set was done, and it became evident that preanalytical variables, such as anticoagulation selection, have a significant effect on outcomes.

Following careful consideration of sample handling, steps must be taken to reduce sample complexity. As described previously, prefractionation of disease samples before further analysis may allow for accurate identification of underrepresented proteins. Havugimana et al evaluated the efficiency of prefractionation measures by carrying out a large-scale proteomics survey on cardiac samples that were prefractionated and those that were not. They found a significant increase in protein coverage in samples that had been prefractionated, highlighting the utility of this experimental technique. As such, prefractionating samples in an effort to reduce sample complexity and improve peptide coverage has become standard procedure. In the final stage of proteomic experiments, spectral data are analyzed and cross-matched to corresponding proteins using publicly available protein sequence databases. However, stringent measures must be put in place to ensure that only relevant spectral data are selected. To address the issue of false-positive detection in MS analysis, filtering may automatically occur as a final step in proteomic experiments. Various groups have opted to include only those proteins matching to at least 2 unique peptides following MS analysis. Furthermore, to elucidate the quality of protein candidate matches, a false discovery rate is determined. Here, a decoy database is created, often consisting of inverted protein sequences, and spectra obtained during the course of MS analysis are searched against a target-decoy database containing forward and reverse protein sequences. The distribution of protein identifications in the target versus decoy database are then used to estimate the peptide/protein false discovery rate. Alternative methods include statistical platforms, such as the Transproteomic Pipeline that uses such algorithms as PeptideProphet and ProteinProphet.
As an example of a large-scale proteomic study, Banfi et al. used a detailed proteomic approach in conjunction with various quality control steps to ensure accuracy of data. They set out to analyze inflammatory changes that occur following coronary artery bypass grafting in patients undergoing cardiopulmonary bypass surgery. In summary, plasma samples were immediately anticoagulated using citrate followed by depletion of albumin and immunoglobulin, 2 of the most-abundant proteins in blood to ensure detection of low-abundance proteins. Proteins were separated using 2DE followed by LC-ESI-MS/MS, with resulting spectra compared against online protein databases (SwissProt/TrEMBL). Stringent criteria were set in place for the inclusion of peptides and led to the high-confidence identification of protease and antiprotease proteins. This study, possessing various levels of quality control, demonstrates how innovations made in the field of proteomic research can offer new therapeutic targets for patients affected by a prevalent cardiac condition.

**Cardiovascular Proteomics**

Careful analysis of the proteomics of the heart has resulted in significant gains in our understanding of heart biology. Nonetheless, our current status of cardiac proteomics somewhat relies on our level of knowledge at this point. For instance, the comprehensive, normal proteome of the *Droso婚纱* heart represents ∼1228 proteins, adult zebrafish hearts express 1375 proteins, the mouse heart appears to consist of 4906 proteins, and at least 3584 proteins are expressed in the human heart. The additional analysis of some of the important subcellular compartments has resulted in the identification of a mouse nuclear compartment of 1048 proteins, 904 proteins in the mouse mitochondria, and 22 proteins in a purified mouse cardiac sarcomeric fraction. Taken together, these proteomes can serve as the reference foundation for numerous studies and insight as we move forward.

Given limited direct access to cardiac tissue, one method to assess cardiac function would be to analyze protein expression in certain noncardiac biofluids. For instance, proteomic analysis of urine might prove useful because this body fluid is easily accessible, most of the low-abundance peptides are generally soluble, and proteolytic degradation is usually complete by the time urine has been produced. The benefits of assessing this proteome in relation to cardiovascular disease were demonstrated by Zimmerli et al. who screened 88 urine samples from patients with coronary artery disease. Application of LC-MS/MS yielded the presence of collagen fragments, with collagen type I and type III fragments being overexpressed in all patient samples, proteins that have been previously associated with coronary artery disease. Urinary proteomics represents a new and growing field of study supported by other research groups that can be used more readily to study cardiovascular disease.

Cardiovascular proteomics also continues to benefit from the analysis of protein samples in tissue taken from a variety of mammalian sources. Cardiac tissue extracted from model organisms, including mouse, rat, pig, rabbit, sheep, and pig, have been performed successfully in proteomic experiments to uncover proteins involved in disease pathogenesis as well as normal mammalian development. Discoveries made in these studies can be translated into human studies in which specific proteins can then be targeted for subsequent analysis. Such a targeted approach in humans has been shown to be successful; however, even exhaustive proteomic approaches using human cardiac tissue in patients with HF, acute myocardial infarction, among others, can be used as a means to uncover innovative biomarkers of the disease state.

However, with the rapid growth of the field of cardiovascular proteomics, it is important to take a step back and consider potential challenges and downfalls that may hamper progression. For instance, sample complexity remains a major concern in any proteomic experiment. One fundamental consideration of any proteomic experiment is the composition of cell type within the sample itself. In the study of cardiovascular disease, tissue generally is obtained from the atria, ventricles, or both, presenting an inherent challenge of sample complexity. However, many improvements have been made in the sophistication of proteomic technology, and these are now being enhanced by changes in the selection of tissue samples. For example, coronary vessels with atherosclerotic disease were subjected recently to 3 separate analyses: direct tissue proteomics, laser microdissection, and frozen sample gel electrophoresis. Interestingly, this study supported the role of direct tissue proteomics in the setting of large-scale proteomic experiments, a technique that has presented challenges in the past. Notably, 1550 unique peptides were identified using direct tissue proteomics when using paraffin blocks as the sample source. Also proving valuable was the application of in-gel trypsin digestion followed by laser microdissection because these platforms improved dynamic range of protein detection, thus enabling increased detection of unique protein matches. The utility of laser microdissection was further assessed recently in a similar model of coronary atherosclerosis. To decrease sample complexity, only the intimal layer of human atherosclerotic arteries was isolated using laser microdissection. Comparison of protein expression levels in the intima of atherosclerotic and preatherosclerotic coronary arteries yielded significantly altered expression of 13 proteins, 3 of which have not previously been associated with this disease state (annexin 4, myosin regulatory light 2 smooth muscle isoform, and ferritin light chain). Identification of proteins in the atherosclerotic proteome may set the stage for establishing disease pathways involved in the progression of coronary artery disease.

**Quantification of Biomarkers**

Several studies have applied the previously mentioned methods to carry out elegant proteomics approaches to cardiovascular disease, and given the need for the early detection of disease, sophisticated quantitative MS-based technologies have emerged to aid with the validation of potential biomarkers. Currently, 4 biomarkers have sufficient evidence of clinical utility to be recommended for clinical use: cardiac troponin I and T used as a current standard biomarker for acute myocardial infarction, B-type natriuretic peptide and N-terminal B-type natriuretic peptide used in the diagnosis of acute as well as chronic HF, C-reactive protein as an inflamma-
tory marker, and D-dimer. Although troponin is an excellent marker for myocardial infarction, the marked increase is not apparent until several hours after the onset of acute coronary syndrome, which emphasizes the need to determine which proteins are elevated at an early stage. Current studies are identifying novel markers and panels of biomarkers that could be used to predict early stages of disease.

Despite the huge potential of biomarkers in helping patient health care and, in turn, longevity, one of the major problems that remains involves accurately identifying biomarkers within patient samples at low, circulating levels in the plasma. Recent advances in quantitative, targeted MS are emerging as a bridge between biomarker discovery and validation. Selected reaction monitoring MS (SRM) is a method that can be used to accurately quantify proteins within a complex sample in a targeted manner (ie, specifically screen for this protein). These technologies use triple quadrupole instrumentation to target specific peptides of identified proteins (ie, proteotypic peptides). Briefly, the selectivity of SRM occurs because a predefined precursor ion undergoes 2 stages of mass filtering. After atmospheric pressure ionization, ions are guided into the first quadrupole, which is set to a very specific narrow m/z window, to selectively isolate the precursor ion or ions of interest. The second quadrupole serves as a collision cell in which the precursor ion is fragmented into specific fragment ions of that peptide and transmitted into the third quadrupole that again acts as a second mass filter, only transmitting a few desired fragment ions that can be detected (termed transitions). These technologies allow the rapid quantification of select proteins and biomarkers with high specificity, sensitivity, and precision from complex solutions and biological fluids. Greater insight can be achieved by taking advantage of the multiplex nature of SRM, which has the capability of detecting up to a 100 different proteins at a varied range of abundance in a single analysis. The caveat with SRM is accurately identifying the most intense and reproducible fragment ions that can identify the target protein or biomarker uniquely. This, however, continues to be facilitated with the development of large inventories of peptides spanning several proteomes collected by the proteomic community, such as those within the PeptideAtlas project (www.peptideatlas.org), which can be used to determine the reproducibility of a particular peptide signature, and the SRMAAtlas (www.srmatlas.org), which to date, contains a repository of validated SRM assays for 1500 yeast proteins and is developing assays for both mouse and human proteins. These advances in SRM assay design will help to continue development for the detection of upregulated proteins in disease states and biomarker verification in plasma.

Pathway Analysis and Integration

With the advent of large data sets, numerous software tools have been developed to analyze the extensive data being generated. One method of evaluating a label-free proteomic data set is to calculate differentially expressed proteins by scoring spectral counts of disease states against healthy states. A common approach is then to take the list of differentially abundant proteins and analyze them based on their functional classification, using several available online tools such as Protein Analysis Through Evolutionary Relationships (PANTHER), Database for Annotation, Visualization and Integrated Discovery (DAVID), The Biological Networks Gene Ontology (BiNGO), and GoMiner, which group proteins into protein families, molecular functions, biological processes, and pathways to discover common characteristics underlying the list of selected proteins. Tools also exist to visualize known protein-protein interactions, such as Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and Cytoscape, which can help to elucidate pathways represented in the differentially expressed proteins.

Proteomics and Beyond

Recent technical advances in proteomics allow us to investigate cardiac muscle to an unprecedented depth. If we can harness these technologies, they will provide not only greater scientific insight into cardiac muscle and related diseases, but also might help us to establish additional markers of disease progression and even identify novel therapeutic targets to increase our ability to manage cardiac patients.

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Disclosures

None.

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