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State-of-the-Art Papers

Proteomic Strategies in the Search of New Biomarkers in Atherothrombosis

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Extensive research has focused on the identification of novel plasma biomarkers to improve our ability to predict cardiovascular events in atherothrombosis. However, classical techniques can only assess a limited number of proteins at a time. Given that plasma contains more than 900,000 proteins, this approach will be extremely time-consuming. Novel proteomic approaches make it possible to compare the expression of hundreds of proteins in several samples in a single experiment. The classical approach consists of separation of proteins on a 2-dimensional gel followed by protein identification with mass spectrometry, although new complementary gel-free techniques are emerging. We can thus compare protein expression in an atherosclerotic plaque with that in a normal artery or study plasma proteins in patients with atherothrombosis as compared with healthy subjects. For such approaches, it is not necessary to study the published data to select potential biomarkers. However, because the number of patients that can be studied with most of these techniques is limited, what is really important is the design of the studies, assessing carefully what kind of patients should be included to obtain valid conclusions. Clinicians should thus play a key role in this design along with the basic scientist. In this article, we review several proteomic strategies carried out by our group and others, and we make a call for collaboration between clinicians and experts in proteomics. This collaboration could greatly increase the likelihood of identifying new prognostic biomarker panels in atherothrombosis and other cardiovascular disorders. (J Am Coll Cardiol 2010;55:2009–16) © 2010 by the American College of Cardiology Foundation

Identifying subjects at risk of developing an acute ischemic event remains one of the great challenges of cardiovascular medicine. Classical approaches, such as the presence of cardiovascular risk factors, are unable to accurately predict cardiovascular events (CVE). In recent years, plasma biomarkers have been the focus of extensive study. Although many potential molecules have been described, the results have not been consistent enough (1), and most of them are not used in clinical practice.

Plasma contains more than 900,000 proteins (2). Given that it takes approximately 10 years from biomarker discovery to the development of a commercial kit (2), testing each of these proteins individually by traditional techniques might take an eternity. Moreover, when several studies about new potential biomarkers with negative results are published, investigators might be discouraged about the usefulness of biomarkers. However, given the large number of proteins present in the plasma, the reporting of negative results for a few potential biomarkers does not invalidate this approach. Moreover, these studies are usually based on individual biomarkers, whereas the use of a panel of biomarkers reporting information of several mechanisms involved in this disorder might be more effective. Therefore we need new methods to screen for novel biomarkers in atherothrombosis.

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New Proteomic Approaches

The standard techniques used for the assessment of proteins in biological specimens, such as enzyme-linked immunosorbent assay, determine only the levels of individual proteins. Proteomic approaches combine 2-dimensional electrophoresis (2DE) and mass spectrometry (MS), allowing hundreds of proteins in a given sample tissue to be assayed simultaneously (3) (Fig. 1). In 2DE proteins are first separated according to their charge by isoelectric focusing in 1 dimension. Then, they are separated further in the second

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Abbreviations and Acronyms

2DE = 2-dimensional electrophoresis

CVE = cardiovascular events

HDL = high-density lipoprotein

HSP = heat-shock protein

ICAT = isotope-coded affinity tags

iTRAQ = isobaric tags for relative and absolute quantification

LC = liquid chromatography

MALDI-TOF = matrix-assisted laser desorption ionization time of flight

MS = mass spectrometry

NSTEACS = non–ST-segment elevation acute coronary syndrome

SELDI-TOF = surface-enhanced laser desorption/ionization time of flight mass spectrometry

sTWEAK = soluble tumor necrosis factor-like weak inducer of apoptosis

dimension according to molecular mass (4). After staining, gels of different samples are analyzed with computer software to detect differentially expressed protein spots. Finally, MS determines the molecular masses of the proteins identifying them (5). This technique requires the conversion of the proteins into gas-phase ions, with various procedures. The ions are separated according to the mass/electrical charge ratio (m/z) with a mass analyzer and analyzed with highly sensitive detectors (5).

Basically, 2 types of MS are used. In matrix-assisted laser desorption ionization time of flight (MALDI-TOF), ionization is achieved by mixing the sample with organic compounds that crystallize to form a matrix. A laser pulse vaporizes the peptides, which are accelerated in an electrical field and are sent to a flight tube, at the end of which the detector is located. For a given electrical acceleration voltage, the time of flight (TOF) to the detector is proportional to m/z. Small molecules fly faster than large ones. The group of peptide masses obtained from its digestion is compared with the theoretical masses of the peptides that would be produced upon digestion of the proteins present in the databases (6), to identify a protein. The second type of spectrometer vaporizes the sample directly from the liquid phase by electrospray ionization or nebulizer (7) with an electrical field to disperse the sample. For this technique, a liquid chromatography (LC) separation step is usually employed before detection to provide a much more reliable protein identification—even with impure protein preparations—than MALDI-TOF. A peptide can then be selected and broken up in a collision chamber. The resulting fragments are sent to the detector, and their masses are obtained. The sequence of the peptide or a short sequence tag is determined by analysis of the fragmentation spectrum. These sequences are then used for database searching. Fragmentation spectra are therefore highly informative and can be powerful tools for characterizing post-translational modifications and for de novo sequencing of unknown proteins.

In addition to 2DE/MS, other platforms have been developed. Gel-free "shotgun" proteomic techniques use LC separation procedures with automated tandem MS and are being applied for the analysis of complex proteomic samples, where a whole proteome is digested with or without prior protein separation. The typical approach is called multidimensional protein identification technology (Fig. 2). This technique identifies proteins in complex mixtures, including basic, highly hydrophobic, or extreme molecular weight proteins, which are difficult to resolve on 2DE gels. It has greater resolution than gel-based approaches but requires rigorous statistical methods, given the large amount of data analyzed.

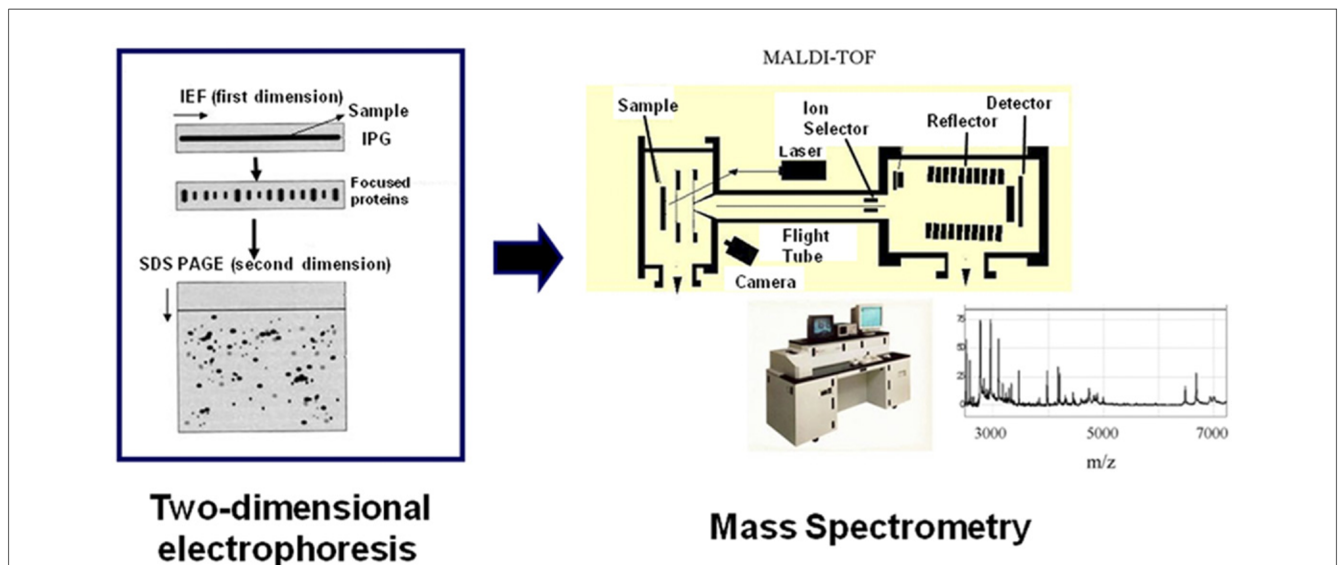

Figure 1 **Classical Proteomic Approach**

(Left) Protein separation by 2-dimensional electrophoresis. **(Right)** Protein identification by mass spectrometry. IEF = isoelectrofocus; IPG = immobilized pH gradient; MALDI-TOF = matrix-assisted laser desorption ionization time of flight; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis. Adapted, with permission, from Vivanco et al. (13).

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