Proteomics in Atherosclerosis

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Atherothrombosis is the underlying cause of several clinical manifestations, such as acute coronary syndromes, cerebrovascular disease, and peripheral artery disease, which together are the leading cause of death in the Western world. Proteins from vascular cells or atherosclerotic plaques that are present in plasma are modified along the different steps of atherosclerotic development and constitute target candidates for vascular research, particularly in the search for novel biological markers of cardiovascular risk. In this review, we summarize proteomic techniques and the most recent results obtained by application of these high-throughput strategies to cardiovascular samples.

Introduction

Atherosclerosis of the coronary arteries, aortic arch, abdominal aorta, and iliac-femoral arteries leads to the most common clinical manifestations of the atherothrombotic process—myocardial infarction, stroke, and peripheral arterial disease. The atherosclerotic lesion starts with the fatty streak formation underlying the endothelium of arteries. Modified low-density lipoprotein cholesterol (LDL-C) deposition on the vessel wall and the recruitment of monocyte/macrophage contribute to the formation of atheroma plaque. Plaque rupture may result in the occlusion of an artery downstream by the formation of a thrombus [1]. During this process, it is possible to distinguish different atherogenic stages in which circulating cells and vessel wall components are involved. The genes or proteins related to these components are directly involved in the pathology and constitute target candidates for vascular research, particularly in the search for novel biological markers of cardiovascular risk. Although classical risk factors (diabetes, abnormal lipid profile, smoking, hypertension) or a previous history of clinical atherothrombosis confer a high probability of future atherothrombotic events, we still need additional prognostic markers to more accurately predict this risk [2].

The absence of perfect correlation between mRNA protein, together with the complexity added by an alternative splicing mechanism and posttranscriptional modifications, have led to the concept that the genome alone is not sufficient to study the functional complexity of an organism. In this sense, proteomic analysis might provide novel information about protein expression in different settings, in general combining two-dimensional gel electrophoresis for protein separation with mass spectrometry (MS) for protein identification. In the past few years, emerging new techniques such as mass spectrometry imaging (MSI) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) have been developed. In regard to vascular research, these recently developed imaging techniques provide an insight into the structure of atheromatous plaque, generating images of the molecular distribution across the tissue using data from MS.

The complexity of atherosclerotic lesions is a considerable obstacle for proteomic analysis. However, some relatively novel techniques, such as laser capture microdissection (LCM), permit the extraction of specific tissue sections, reducing the sample heterogeneity by dissecting specific cell types. Nevertheless, although this technique is capable of analyzing different regions of atherosclerotic lesions in an independent way, the subsequent proteomic analysis is limited due to the reduced amount of protein that these techniques can obtain.

The use of different proteomic strategies allows us to study the vascular proteomes of cells, tissues, and plasma. Furthermore, the evolution of classical proteomic approaches and the new proteomic tools will probably represent a considerable advance in the search for proteins involved in cardiovascular diseases.

Techniques in Proteomic Analysis

The techniques in proteomic analysis are usually divided according to the use of polyacrylamide gels or free-gel

Table 1. Advantages and disadvantages of major proteomic approaches

Technique	Principle	Advantages	Disadvantages
Gel-based techniques			
Two-dimensional electrophoresis	Protein separation in polyacrylamide gels	Analysis of all proteins present in the sample	Reduced dynamic range, semiquantitative, over 100 μg required, low throughput (1 sample per gel)
2D-DIGE	Pre-electrophoretic fluorescent protein labeling	Use of fluorescent dyes increases the sensitivity of the technique, allows real quantification of differences in protein expression	Reduced dynamic range, over 50 μg required, low throughput in spite of 3 samples per gel
Free-gel techniques			
LC-MS/MS	Alternatives to gel electrophoresis for the protein separation step	Protein separation and protein identification steps are coupled, high sensitivity in protein separation, reduction of sample complexity	End point analysis because it uses MS as detector, needs robust data mining; expertise (more complex instrument)
SELDI-TOF	Alternatives to gel electrophoresis for the protein separation step	Whole samples can be applied directly, reduction of sample complexity	Lower resolution than MALDI-TOF, unsuitable for high molecular weight proteins
Imaging techniques			
MSI	Mapping of the complex molecular pattern across a tissue sample. It can be correlated to particular histologic regions	Spatial distribution of protein in tissue sample is provided; pharmaceutical agents and resulting metabolites within tissue sections can also be obtained, opening new possibilities for the measurement of protein changes in specific tissues after systemic drug administration	Only molecules that can be ionized will be detected; very complex samples produce ion suppression effects, reducing spectra quality
TOF-SIMS	Mapping of the complex molecular pattern across a tissue sample. It can be correlated to particular histologic regions	Provides a molecular map of lipid distribution across the tissue, very high lateral resolution, and matrix-less preparation	Reduced mass range

2D-DIGE—two-dimensional difference gel electrophoresis; LC-MS/MS—liquid chromatography coupled with tandem mass spectrometry; MALDI—matrix-assisted laser desorption/ionization; MSI—mass spectrometry imaging; SELDI—surface-enhanced laser desorption ionization; SIMS—secondary ion mass spectrometry; TOF—time of flight.

proteomic tools. In addition, there are imaging techniques that provide additional spatial information about the distribution of the different molecules present in the arterial wall. The advantages and disadvantages that characterize these proteomic approaches are collected in Table 1.

Two-dimensional gel electrophoresis

Most cardiovascular studies reported to date have employed two-dimensional gel electrophoresis (2DE) with immobilized pH gradients to separate the proteins of the sample. 2DE involves the separation of solubilized proteins in the first dimension according to their charge (isoelectric point) under denaturing conditions. Then, in the second dimension, they are separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to their relative molecular weight. Because the charge and the size of proteins are independent parameters, the separation results in a 2DE profile of protein distribution.

The 2DE method has some variations that have been successfully applied by several investigators, such as two-dimensional difference gel electrophoresis. This technology is based on the introduction of different fluorescent dyes to label the proteins before their separation. This characteristic enables researchers to separate different conditions in the same gel, minimizing gel variations. The overlay of corresponding images makes the comparison between them easier and makes possible the improvement of the statistical analysis of the previous results. This technology is a reliable method to define quantitative protein differences. An example of this proteomic tool can be seen in Figure 1.

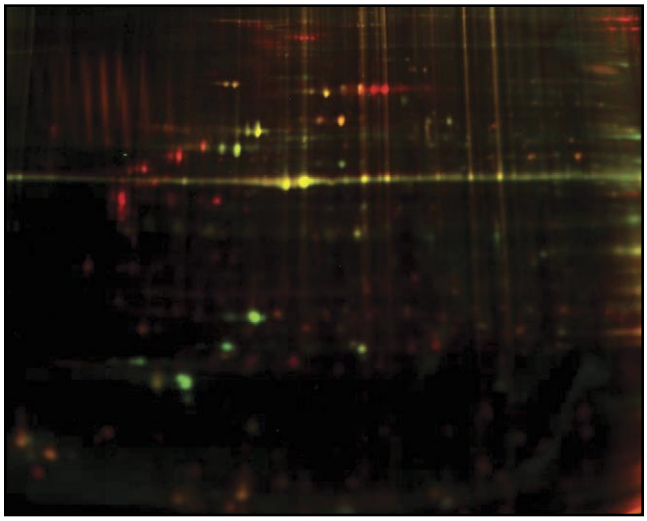

Figure 1. Comparison of the protein profile found between aortas from apolipoprotein E–deficient mice versus wild-type mice. Pooled aortas from five animals of each group were homogenized and resuspended in a lysis buffer. After protein precipitation, 50 µg of protein was labeled with cyanine (Cy) 3 and Cy5 dyes. The experiment introduces an internal standard labeled with another dye (Cy2). The isoelectric focusing was performed using 4-cm to 7-cm immobilized pH gradient strips. The result after two-dimensional gel electrophoresis separation is shown. Proteins labeled with Cy3 dye will appear in green and correspond to apolipoprotein E samples. Proteins labeled with Cy5 dye will appear in red and correspond to wild-type animals. The proteins found in both conditions will appear in yellow. The proteins detected in both cases with significant difference in the expression levels are candidates to be identified by mass spectrometry. These identifications are now being studied.

Free-gel proteomic approaches

Another proteomic approach is based on the use of alternatives to gel electrophoresis for the protein separation step. In these cases, the sample requires a specific treatment, usually by sample enzymatic digestion after homogenization, followed by a subsequent separative step performed using a high-resolution chromatographic column. In liquid chromatography coupled with tandem MS (LC-MS/MS), peptides are fractionated in base to their hydrophobicity prior to the MS analysis in order to reduce sample complexity. Due to its extraordinary sensitivity, this approach has been selected for use with laser microdissected samples or paraformaldehyde/paraffin-embedded analysis by direct tissue proteomics [3••]. Also, it has been described as a way to circumvent problems associated with the isoelectric focusing step, combining a standard SDS-PAGE followed by protein identification with LC-MS/MS.

The surface-enhanced laser desorption ionization TOF MS (SELDI-TOF MS) platform is a developing technology that is employed in proteomic analysis of fluid samples. With this platform, proteins of the sample are fractionated on a solid-phase protein chip surface (protein chips) before the MS analysis. The proteins that are retained in the protein chip have specific chemical properties and only these proteins will be analyzed by MS. This protein chip is placed directly in the SELDI-TOF spectrometer, where the proteins are ionized with a laser and their molecular masses are measured with a TOF MS detector. SELDI-TOF platforms have been recently used in cardiovascular research with the aim of finding biological markers in human plasma [4] and in atherosclerotic tissue–conditioned media [5•].

The characterization of phosphoproteins is one of the main areas in proteomic research, including the application of phosphoserine and phosphotyrosine antibodies. A standard methodology for phosphorylation studies relies on peptide fractionation and its further enrichment using immobilized metal affinity chromatography (IMAC) in order to perform the phosphopeptide isolation. Immobilized metal ions, such as iron(III), bind phosphorylated peptides and some phosphoproteins with high selectivity. Iron(III) IMAC columns have been used in combination with reverse-phase high-performance liquid chromatography and matrix-assisted laser desorption/ionization (MALDI) MS, and with LC-MS/MS for phosphopeptide purification and characterization at picomole and subpicomole levels. Several methods for phosphoprotein purification have been described in cell and tissue samples [6••,7]. Duran et al. [8] have described the strategies used to characterize the differential phosphorylation states detected in the two heat shock protein 27 (HSP27) isoforms identified in atherosclerotic plaque supernatants by application of direct LC-MS/MS and further combination of IMAC columns with LC-MS/MS.

Another methodology used to characterize these post-transcriptional modifications is based on the use of strong cation exchange columns for peptide fractionation, where the peptides are eluted according to their charge (positive peptides are retained in the column). The phosphate group subtracts one positive charge from the peptide; therefore, phosphopeptides will elute before nonphosphorylated peptides. The following phosphopeptide enrichment can be performed using titanium dioxide (TiO_2) columns instead of IMAC columns. TiO_2 binds phosphorylated peptides with a very high binding affinity, but it also can bind nonphosphorylated peptides. The phosphopeptide binding can be more selective using 2,5-dihydroxybenzoic acid, which competes with nonphosphorylated peptides for the TiO_2 binding [9].

Imaging techniques (MSI and TOF-SIMS)

In the past few years, there have been several attempts to directly couple the tissue sample with a mass spectrometer. To generate images from the MS data, the spectrometers that are most frequently used are the MALDI-TOF and TOF-SIMS. The basis of the technique is to raster the whole tissue surface by the laser or ion bean. The resulting spectra (intensity vs mass-to-charge ratio) are recorded to each position (x, y).

Molecular imaging of tissues by MALDI-TOF is a powerful proteomic tool that provides information on spatial

distribution of proteins and other components of the tissue. MSI technique has been used in studying several pathologies, although the applications to vascular research have not been very successful. Several components of atherosclerotic tissue sample, such as lipid molecules, salts, and very common proteins in high concentrations, may mask potential candidates. New methods for the sample preparation are required to improve the results of the technique.

TOF-SIMS has been more successful than MSI for the application to vascular tissues. Tissue imaging with TOF-SIMS has recently been employed to generate molecular ion images of human atherosclerotic plaques [10] and rat aortic vessels [11]. This technique provides images mainly of hydrophobic molecules; therefore, several lipids might be overrepresented in the spectra whereas other abundant components of the vascular tissue might be completely absent [12]. These molecular maps of lipid distribution across the tissue could be very useful in cardiovascular research overall and in the study of atherosclerosis lesions in particular, as lipids play an important role in plaque development.

Recent Results Obtained by Application of Novel Proteomic Techniques to Cardiovascular Samples

The sampling is a critical step in any kind of proteomic analysis. For example, if the objective of the study is to find circulating markers in blood, the tissue secretome or plasma should be investigated directly. However, if the interest of the study is to understand the mechanism of the atherogenesis, the cell or tissue extracts would be preferred [1]. In addition, the type of sample determines the proteomic tool that must be used in the analysis. In this section, we report recent results obtained by proteomic application to different kinds of samples: cells, tissues, and plasma.

Cell and tissue

Proteomic analysis can be carried out on different cell constituents of atheroma plaques. This alternative involves the use of cell cultures resembling the phenotypic conditions of atherosclerosis lesions. In this sense, it is possible to study endothelial cells, vascular cells, and platelets.

Vascular smooth muscle cells (VSMCs) play a crucial role in atherogenesis. When a vessel is injured, serum comes into contact with VSMCs. Hence, growth factors, cytokines, and many others soluble components probably interact with plasma membranes of these cells, and this may activate intracellular signaling cascades. These events may initiate the proliferation and migration of VSMCs. Recently, the events involved in the activation of VSMCs using a classic proteomic approach have been described [13]. This work focused on the modulation of tyrosine phosphorylation, which occurs in cell activation by serum or by single growth factors, such as insulin-like growth factor 1 or platelet-derived growth factor–BB. A comparison of profiles from two-dimensional polyacrylamide gel electrophoresis of quiescent and activated VSMCs has revealed several differences in protein expression, among them important changes in the phosphorylation of chaperones, suggesting their crucial role in VSMC activation. An extensive protein profiling of human VSMCs has also been recently published [14]. The study examined VSMCs from human internal mammary arteries obtained from patients displaying symptoms of coronary disease. This work revealed two protein maps of human VSMCs, one of the proteome (the intracellular proteins) and the other of the secretome (the proteins that are secreted by the cells to the media), providing a basis for vascular biology investigations.

Endothelial cells are other components of atheroma plaques. These cells form a continuous monolayer lining the inside faces of all blood vessels and present the ability to selectively control vascular permeability. Human umbilical vein endothelial cells are currently the most commonly used in vitro model. A differential proteomic approach using these cells under basal quiescent conditions and activated by stimulation with proinflammatory cytokines, such as interleukin-1β, has been recently published [15]. The analysis of a series of 2DE gels allowed the selection of 233 proteins and showed that 70% of them had an increase and 30% a decrease in the expression levels in activated cells. Subsequent analysis of 35 altered proteins was made by MALDI-TOF MS. The comparative analysis carried out in this work shows the usefulness of a proteomic approach in identifying quantitative and qualitative variations in protein levels associated with specific vascular dysfunctions.

Platelets play a pivotal role in atherothrombosis after coronary artery plaque rupture. In a recent study, microarray and proteomic studies on platelets from individuals with an "extreme end" response phenotype provided further insight into key regulators of platelet function [16]. More recently, highly enriched, human platelet, dense granule fractions have been analyzed using two proteomic methods. Among the identified proteins, the authors focused on the analysis of 14-3-3zeta, which is secreted after platelet activation. It has also been detected in sections of human abdominal aorta of patients with aneurysm and in atherosclerotic plaques [17].

In addition to vascular or circulating cells, other cells present in the organism can also release potential biomarkers into the circulation. Xu et al. [18] focused on the adipocyte secretome, identifying proteins released from adipocytes and present in human plasma. The authors identified adipocyte fatty acid–binding protein as a circulating biomarker closely associated with obesity and metabolic syndrome, two risk factors for atherothrombosis. Therefore, the measurement of serum concentrations of adipocyte fatty acid–binding protein might be useful for clinical diagnosis of obesity-related metabolic and cardiovascular disorders.

Proteomic analysis can be performed directly on tissue samples. In this respect, atherosclerotic tissue can be homogenized and then analyzed [19]. However, this kind

of analysis can be difficult due to the heterogeneous cellular (VSMCs, macrophages, other inflammatory cells) and molecular (lipid content, calcification) composition of atherosclerotic plaques. To circumvent this problem, tissues can be laser microdissected to study very specific regions or cell types within a tissue section of the atheroma plaque. Although this strategy is quite straightforward, tissue amounts constrain its use in combination with 2DE. LCM is compatible with direct tissue proteomics, which can be used to identify proteins directly from formalin-fixed, paraffin-embedded tissue samples. Bagnato et al. [3••] used this proteomic approach to analyze human coronary vessels in various stages of atherosclerosis. The analysis of 35 human coronary atherosclerotic samples allowed identification of a total of 806 proteins as a result of combination of three different sample treatments (direct tissue proteomics, LCM, and gel trypsin digestion).

Recently, two-dimensional difference gel electrophoresis technology has been used to analyze rat heart tissue in order to identify proteins that are involved in ischemia-reperfusion injury. The comparison of normal, ischemic, and ischemic-reperfused rat hearts showed eight areas that changed significantly. Five of them were identified by MALDI-TOF MS as protein disulfide isomerase, a 60-kD heat shock protein, and elongation factor 2 [20].

Another approach is to study the secretome of vascular tissue, which includes all proteins that are secreted or released by cells or tissues in the extracellular compartment. The goal of characterizing the secretome of the atherosclerotic plaque is to find novel biological markers that could be related to cardiovascular diseases. By means of using this and other methodologies, there have been some potential biomarkers of atherosclerosis identified in the past few years (Table 2). Among these, we previously observed that HSP27 was released in smaller amounts by atherosclerotic plaques than by healthy arteries. This was due to the proteolysis of the protein in the atherosclerotic environment [21]. In addition, HSP27 can be modified posttranslationally by phosphorylation. We [8] observed that one specific phosphorylated isoform of this protein is decreased in the secretome of the lesion. Recently, using another proteomic approach, Matt et al. [22] identified one isoform of phospho-HSP27 by studying aortic aneurysms associated with bicuspid aortic valves and tricuspid aortic valves. This work shows that HSP27 is decreased in bicuspid aortic valves compared with tricuspid aortic valves. However, the putative mechanisms by which HSP27 isoforms could be involved in the atherosclerotic process are still unknown.

SELDI-TOF platforms have recently been used in cardiovascular research with the aim of finding biological markers in atherosclerotic tissue–conditioned media [5•]. This study revealed an 18.4-kD peak released in lower amounts by carotid plaques than normal endarteries, which was identified as soluble tumor necrosis factor–like weak inducer of apoptosis (sTWEAK). To confirm that sTWEAK was the protein of interest, Western blot and enzyme-linked immunosorbent assays were performed. Subsequent measurement of sTWEAK in plasma showed a reduced concentration in patients with carotid stenosis compared with healthy controls matched by sex and age. Furthermore, in a test population of 106 asymptomatic patients, sTWEAK concentrations negatively correlated with the carotid intima-media thickness. These results suggest that sTWEAK may be a biomarker of atherosclerosis.

Plasma

The plasma proteome is very interesting from a clinical point of view because it can provide the physiologic state of the different tissues in the body. However, this analysis becomes difficult due to its composition and the presence of very high concentrations of some proteins, like albumin and immunoglobulins (on the order of g/L), which mask the underrepresented ones. Nine major proteins represent 90% of the plasma proteome, and 12 other proteins account for an additional 9%. The challenge is thus to reach the "deep proteome" comprising the remaining 1% of the plasma proteome [23]. Several approaches can be applied to analyze the plasma proteome directly or after a fractionation step. Depletion of abundant proteins, SELDI-TOF platforms, protein precipitation, and protein arrays have been used with this aim.

Proteomic analysis can also be used to test the effect of drugs in cardiovascular patients. In a recent article, proteomic analysis was applied to detect modifications in the protein map of plasma after statin treatment on hypercholesterolemic patients [24]. The 2DE analysis followed by MS provided the authors the identification of three fibrinogen γ chain isoforms (FGG), with different expression levels in both groups. In addition, the expression of apolipoprotein A-IV and three haptoglobin isoforms was higher in the hypercholesterolemic patients. Statin treatment modified the plasma expression of FGG chain isoform 1, fibrinogen β chain (FGB) chain isoforms 1 and 2, vitamin D binding protein isoform 3, apolipoprotein A-IV, and haptoglobin isoform 2. Recently, a proteomic study regarding the relationship between vitamin D binding protein and aspirin resistance in coronary ischemic patients was recently published [25]. The expression of one isotype of the FGG chain and three isotypes of haptoglobin was increased in plasma of aspirin-resistant patients. In this work, three vitamin D binding protein isotypes were increased in aspirin-resistant patients.

Searching for biological markers becomes one of the main goals in the study of any pathology. The most extensively studied potential biomarker of cardiovascular risk to date has been C-reactive protein (CRP). In addition to CRP, a number of different proteins, mainly involved in the inflammatory process, have been studied in recent years as potential candidates to predict risk factor. Among these are CD40L, monocyte chemoattractant protein-1, adhesion molecules, myeloperoxidase, and several interleukins.

Table 2. Proteins related to atherosclerosis identified in proteomic studies

Protein identified	Protein change	Protein identified	Protein change
ATT	Increased	Dissociation inhibitor-α	
Apolipoprotein AI	Increased	Interferon-γ inducible	
γ-Chain of fibrinogen	Increased	Lysosomal thiol reductase	
Heavy chain of IgD	Increased	Cathepsin D	
Serum albumin	Increased	A-FABP	Increased
Ferritin light chain	Increased	Integrin-IIβ	Decreased
Apoptosis-linked gene-2	Increased	Macrophage capping protein	Increased
HSP27	Decreased	Superoxide dismutase	Increased
Metalloproteinase-1		Catalase	Increased
Collagenase		Platelet-activating factor acetylhydrolase	Decreased
Plasminogen activator inhibitor-1		β2-microglobulin	Increased
Peroxiredoxin 1		AnnexinV	Increased
HSP90	Increased	EnolaseI	Increased
Cyclophilin B	Increased	Glutation-S transferase	Increased
Cathepsins D, L, S	Increased	sTWEAK	Decreased
Proteoglycans		Laminin-binding protein	Increased
Paraoxonase		Stomatin-like protein 2	Increased
Apolipoprotein D	Decreased	Signal sequence receptor	Increased
Cathepsin H		Tyrosine 3-monooxygenase	Increased
Cofilin 1		Fortilin, tumor protein	Increased
CapG (gelsolin family)		Tropomyosin isoform	Increased
Gelsolin		Gads	
CapZ			
HSP60	Increased		
HSP70	Increased		
Protein disulfide isomerase	Increased		
Elongation factor EF-1	Increased		
Vimentin	Increased		
Actin	Increased		
Plasminogen activator inhibitor-2	Increased		
Liver LKB-interacting protein	Increased		
Rho GDP	Decreased		

A-FABP—adipocyte fatty acid–binding protein; ATT—α1-antitrypsin; Gads—Grb-2-like adaptor protein; GDP—guanosine diphosphate; HSP—heat shock protein; sTWEAK—soluble tumor necrosis factor-like weak inducer of apoptosis.

SELDI-TOF platforms have recently been used in cardiovascular research with the aim of finding biological markers in human plasma. This platform has been used to screen for differentially expressed proteins in plasma samples from patients with peripheral arterial disease. In patients with peripheral arterial disease, circulating β_2-microglobulin is elevated and correlates with the severity of the disease independent of other risk factors [4]. These findings might provide a new potential biomarker of cardiovascular disease measured in human plasma using the SELDI-TOF platform.

Conclusions

Large-scale technologies are powerful tools to study the pathways of complex diseases such as atherosclerosis [26]. Proteins are the major effectors of most biological processes and constitute the most suitable molecules for use as biomarkers as well as targets for disease treatment. Incorporation of proteomics in cardiovascular research provides the necessary tools to identify and characterize complex protein changes associated with cardiovascular dysfunctions.

The new imaging techniques represent a powerful tool to analyze the vascular tissue because of the spatial distribution of proteins and other molecular components of the vessels that these techniques provide. SIMS-TOF imaging is a promising technique for the analysis of the surface of the vessel wall that allows identification and localization of unknown surface molecules, enabling not only molecular mapping but also the discovery of novel components of the tissue, both key aspects of molecular biology.

Identification of novel biomarkers will also help us to assess cardiovascular risk. However, it is still necessary to develop new methods that permit the detection of more proteins with high predictive and prognostic characteristics that can be translated into clinical practice. In the future, proteomic analysis will identify novel biological markers that will help to target vulnerable patients and monitor the beneficial effects of pharmacologic agents.

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Disclosures

No potential conflicts of interest relevant to this article were reported.

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