Integrated Biomarker Discovery and Validation Implicates the Complement Pathway in Early Atherogenesis*

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There is a pressing need to identify reliable biomarkers that can distinguish among the early, intermediate, and late stages of atherosclerosis, the leading cause of death in industrialized societies. One powerful approach is the use of quantitative tandem mass spectrometry (MS/MS) to identify candidate protein markers in plasma. Advantages of this approach include the ability of multiplexed MS/MS to identify and quantify a large number of protein, the availability of plasma from clinical and translational studies, and the established importance of plasma proteins (e.g., apolipoproteins such as APOB, the major protein of low-density lipoprotein, the “bad” form of cholesterol) in the pathogenesis of atherosclerosis.

Despite impressive technical advances over the past 20 years in MS and other quantitative approaches to multiplexed proteomics, there has been a remarkable dearth of identification of new biomarkers that can serve as robust, reliable guides for improving diagnosis, directing therapy, and providing insights into the underlying mechanisms of cardiovascular disease (CVD). As first proposed in a classic paper by Rifai et al. (1), a major challenge is to develop an integrated approach that links the identification of biomarkers to their validation in translational studies. These workers proposed 4 key steps in biomarker development: discovery—identification of candidate biomarkers; qualification—demonstration of differential abundance in plasma (or other biological samples); verification—assessment of a biomarker’s specificity; and validation—determining the sensitivity and specificity of a biomarker in multiple clinical populations.

In this issue of the Journal, Martínez-López et al. (2) integrated several stages of this approach to identify candidate protein biomarkers for atherosclerosis. For the discovery phase, they focused on the analysis of fatty streaks and fibrolipid lesions harvested from human aortic tissue. By selecting early atherosclerotic lesions, they clearly were targeting biomarkers that linked to disease onset. Another important strength of their approach was the use of abdominal aortic tissue harvested from organ donors, thereby avoiding many of the problems associated with postmortem changes and use of autopsy material for biomarker identification. The authors also used a well-validated MS/MS approach to quantitative multiplexed proteomics together with conservative approaches to protein identification and statistical analysis of changes in relative protein abundance.

Focusing on fatty streak (FS) and fibrolipidic (FL) lesions in the media and intima layers, their approach identified more than 3,000 proteins in early atheros. About 20% of the proteins were differentially expressed in lesions relative to their levels detected in normal media layers. Among the most highly significant functional categories within FS and FL were pathways associated with fibrinolysis, platelet biology, and the complement system. Consistent with
the well-established roles of lipoproteins in all stages of atherogenesis, several categories relating to lipid transport, HDL remodeling, and lipoprotein metabolism were also up-regulated.

Among these pathways, the increased abundance of proteins linked to the complement system was most striking. In both of the intimal and medial lesions, large increases were seen in proteins implicated in regulation of complement by both the classical and alternative pathways. Importantly, the authors confirmed the up-regulation of several complement proteins in lesions by immunohistochemistry, immunoblotting, and enzyme-linked immunosorbent assay. These observations provide intriguing evidence that the complement system is up-regulated in early human atherosclerotic lesions and, hence, may serve as a potential marker to identify people at risk for CVD.

Despite these strengths, one shortcoming is the relatively small number of lesions analyzed, along with an experimental design that lacked a validation cohort. However, there are critical technical challenges, primarily the inherent difficulties in harvesting well-preserved arterial tissue from humans, that often hinder a thorough probing of the complex proteome of normal versus diseased vascular tissues. The authors worked to overcome these limitations and determine if the complement system might indeed have pathophysiological relevance to early atherogenesis. For this, Martínez-López et al. (2) focused on C5, which is proteolyzed by C5 convertase to C5b—the first component of the membrane attack complex formed by activation of either the classical or alternative pathways (3). Immunoblotting and enzyme-linked immunosorbent assay confirmed increased levels of C5 and C5b in lesions, and immunohistochemistry demonstrated colocalization of the uncleaved substrate (C5) with the membrane convertase complex. In contrast, the authors detected no increase in C5 mRNA in tissue, consistent with the likely origin of C5 from plasma. Taken together, these observations fulfill the qualification and verification steps proposed by Rifai et al. (1). Importantly, they suggest a potential role for C5 or its cleaved products in early atherogenesis.

A key issue is if these findings are clinically relevant. To address this question, Martínez-López et al. (2) focused on plasma, which is readily available and widely used for assessing patients’ cardiovascular risk. Using 2 large, independent cohorts of apparently healthy subjects—1 composed of men only (the PESA [Progression of Early Subclinical Atherosclerosis] study) (4) and the other of about equal numbers of men and women (the NEFRONA [Observatorio Nacional de Atherosclerosis en NEFrologia, a prospective, multicenter observational study of renal disease in Spain]) (5)—the authors found that plasma C5 levels predicted subclinical atherosclerosis as quantified using 2- and/or 3-dimensional vascular ultrasound. Plasma C5 levels also independently predicted coronary artery calcium (CAC) scores determined by CT scan in PESA (4). This is important because CAC is widely regarded as one of the best methods for assessing the risk of CVD in subjects who are at intermediate risk for clinical events. Although these observations suggest that C5 is a promising candidate biomarker for identifying early atherosclerotic CVD, the specificity for C5—which has roles in numerous conditions and host immune responses—was not addressed.

Another key issue is whether inhibiting C5 pharmacologically might affect cardiovascular risk. Two large clinical trials have investigated the effect of pexelizumab—an anti-C5 monoclonal antibody—on infarct size in patients with ST-segment elevation myocardial infarction (MI) undergoing primary percutaneous coronary intervention. The COMMA (COMplement inhibition in Myocardial infarction treated with Angioplasty) trial (6) found no effect on infarct size with pexelizumab, but the study did observe a significant reduction in death on follow-up. In contrast, the larger APEX-AMI (Pexelizumab in Conjunction With Angioplasty in Acute Myocardial Infarction) study (7) found no difference in mortality rates through day 30 between the pexelizumab and placebo groups. However, it is important to note that both studies centered on the consequences of tissue injury during acute ischemia-reperfusion, a late complication of advanced atherosclerosis. That endpoint is clearly temporally and pathophysiologically distinct from the early stage of lesion development studied by Martinez-Lopez et al. (2) Thus, the relevance of C5 as a reliable biomarker of early atheroma requires further investigation.

Furthermore, an additional, important limitation of the study by Martínez-López et al. (2) was the use of surrogate markers for quantifying early atherosclerosis. Elevated levels of candidate biomarkers relative to surrogate markers of atherosclerosis do not provide strong evidence of a link to the development of CVD events. Observational studies demonstrate that fatty streaks and fibrolipid lesions are common in older subjects without known CVD. If elevated levels of C5 predicts progression to more advanced lesions (e.g., by quantifying CAC) or CVD events in apparently healthy subjects, they would provide a powerful new tool for assessing CVD risk. It would also raise the possibility that pexelizumab therapy could help prevent the progression of early atherosclerotic disease.
Thus, in a comprehensive study, the authors demonstrate the power of using the 4-pronged approach to biomarker discovery proposed by Rifai et al. (1): discovery, qualification, validation, and translational studies in multiple populations.

**REFERENCES**


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