Abstract—Inflammation plays a key role in atherosclerosis. A number of different biomarkers of inflammation are measurable in blood. These include cytokines, chemokines, soluble adhesion molecules, and acute-phase reactants. The first 3 groups of molecules are not routinely measured in clinical laboratories. The acute-phase reactants include C-reactive protein (CRP). This analyte is stable and is readily measured by several commercial high-sensitivity (hs) assays, and numerous clinical studies have demonstrated its usefulness as an atherosclerotic risk marker. For these reasons, CRP is currently the inflammatory marker of choice. Comparison of commercial hsCRP assays has demonstrated that many are capable of imprecision of $<7\%$ at a CRP concentration of $<1$ mg/L. Method comparability studies have shown good agreement among some commercial hsCRP assays in terms of quartile assignments, whereas additional standardization efforts are necessary for others. The first goal of standardization is to develop a secondary reference material with a CRP of $\approx 4$ mg/L. This material can be used to assist diagnostic vendors in calibrator value assignment and assay harmonization. Another project is to define clearly what performance characteristics are necessary for hsCRP assays. Preliminary estimates based on the biological variability of CRP indicate that the total allowable analytical error is between 15% and 32%. The former requirement can be met with accuracy and imprecision of $\leq 5\%$ and the latter requirement with accuracy and imprecision of $<11\%$. If 2 hsCRP results are averaged, then the imprecision requirements are relaxed slightly. Clinical validation of these performance requirements is also required. (Circulation. 2004;110:e572-e576.)

Key Words: AHA Scientific Statements • atherosclerosis • inflammation • risk factors

Inflammation plays a key role in the pathophysiology of atherosclerotic disease. A number of inflammatory markers that are measurable in blood have been investigated for their ability to predict the risk of future atherosclerotic events. These markers can be divided into 3 groups of molecules: cytokines and chemokines, soluble adhesion molecules, and acute-phase reactants. A number of preanalytical and analytical issues including specimen type and stability, assay imprecision, commercial availability, and standardization are reviewed here for selected members of each of these 3 groups of markers.

Cytokines and Chemokines
A large number of cytokines and chemokines have been described. Among those that have been investigated for a relationship to atherosclerosis are interleukin-1β (IL-1β), IL-6, IL-8, IL-10, tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein (MCP-1). All of these molecules are relatively unstable in serum. Serum or plasma must be rapidly separated from the cellular components of blood and assayed rapidly or frozen to prevent analyte degradation. Typically, these assays are performed in re-
search laboratories via ELISA techniques. For some cytokines, more automated methods are becoming available for research. An automated microplate system for IL-6 has been described.\(^3\) Chemiluminescent assays for IL-6, IL-8, and TNF-α are available on an automated immunoassay analyzer for research use only in the United States.\(^3\) Multiplex assays for several cytokines have also been described.\(^4\) This methodology shows great promise, but at present it is still a research tool rather than a routine part of laboratory medicine practice. Most of the cytokine assays that have been used show imprecision, as assessed by a coefficient of variation between 2% and 15%, according to the concentration of analyte being measured.

Two general limitations of many cytokine assays are the lack of a sufficiently low limit of quantification for use in apparently healthy subjects and problems with assay standardization. Many of the commercial cytokine assays are designed to quantify the relatively high concentrations of cytokines encountered in acute inflammatory processes. For cytokines to be useful for atherosclerotic risk prediction, the assay must be able to accurately and precisely quantify cytokine levels within the reference interval (ie, the concentrations encountered in an apparently healthy population) so that subjects can be divided into tertiles, quartiles, or quintiles. Standardization of cytokine and chemokine assays is problematic. Although international standards exist for several cytokines, including IL-1β, IL-6, IL-8, and TNF-α, standardization of cytokine immunoassays is a difficult process.\(^5-8\) One reason for this may be that the nature of the different monoclonal antibody pairs used for each assay does not permit comparable recognition in individual patient samples.\(^7\)

### Soluble Adhesion Molecules

A number of soluble adhesion molecules have been examined as possible markers for atherosclerotic risk. E-selectin, P-selectin, intracellular adhesion molecule-1 (sICAM-1), and vascular adhesion molecule-1 (sVCAM-1) are some of the better-studied molecules. Little information is available about preanalytical conditions affecting their recovery. Typically, like most cytokines, they are unstable unless frozen. ELISA methods generally are used to measure these analytes in research laboratories. The availability of commercial kits is limited. These analytes are poorly standardized. Overall, these limitations preclude their consideration for routine use in the clinical laboratory.

### Acute-Phase Reactants

Acute-phase reactants are a group of proteins that are synthesized by the liver in response to cytokines, primarily IL-6. Fibrinogen, serum amyloid A (SAA), and C-reactive protein (CRP) are 3 acute-phase reactants that have been best studied as inflammatory markers of atherosclerosis.

Fibrinogen is a clotting factor produced by the liver that has been widely studied as an inflammatory marker of atherosclerotic events. It usually is measured in sodium citrate plasma, although EDTA plasma can be used for some assays. Fibrinogen is relatively unstable, and plasma should be separated from cells and frozen as soon as possible if not analyzed immediately.\(^9\) Two general classes of methods are available: immunoassays and functional assays. Many automated commercial methods are available, and most hospital laboratories have the capability to measure fibrinogen. The imprecision of most assays is in the range of 4% to 8%. Despite that several studies have indicated that fibrinogen is a good inflammatory marker for atherosclerotic risk and the assay is widely available, difficulties with assay standardization makes this analyte unattractive for routine clinical use as a marker of atherosclerotic risk.\(^10\) An international plasma standard (World Health Organization [WHO] 2nd International Standard [IS] Fibrinogen, Plasma 98/612) is available for fibrinogen.\(^11\) Because several different methodologies are used, however, standardization is problematic. The fibrinogen concentration can be estimated from the clotting curve of the prothrombin time on automated photo-optical coagulometers. These results can be significantly higher than values derived from Clauss measurements, especially in patients receiving oral anticoagulants, in some patients with documented hypo- and dysfibrinogenemia, and in patients with high fibrinogen concentrations.\(^12,13\) The Clauss method can underestimate the fibrinogen concentrations in the presence of hirudin peptide 54-65 and fibrinogen fragment E.\(^13\) Assignment of fibrinogen values to commercially available plasma quality control materials also can be problematic, and significant variability from instrument to instrument exists.\(^14\) The use of a single type of functional assay, or perhaps better yet the use of immunoassays directed toward a specific epitope, may facilitate standardization of fibrinogen assays and improve the feasibility of use of this marker.

SAA protein is another acute-phase reactant that has been examined as a risk factor for atherosclerotic events. SAA in serum samples is stable for 8 days if refrigerated.\(^15\) A single commercial nephelometric assay is available in the United States for research use only. The imprecision of this assay is <9%.\(^16\) An international standard for SAA (WHO 1st IS 92/680) has been established.\(^17\) Multiple phenotypes of SAA are known to exist that have the potential to complicate future immunoassay standardization efforts for this analyte.

### High-Sensitivity CRP

High-sensitivity measurement of CRP (hsCRP) has received a great deal of attention recently for use as an atherosclerotic risk marker. CRP is a 120-kDa pentamer that can be measured by a variety of methods including radioimmunoassay, immunonephelometry, immunoturbidimetry, immunoluminometry, and ELISA. High sensitivity is loosely defined as having sufficient precision at low concentrations of CRP to allow stratification of the results from apparently healthy subjects into tertiles, quartiles, or quintiles. Practically speaking, this translates into a lower limit of quantification of ≥0.3 mg/L and assay imprecision of <10% at a CRP concentration <0.5 to 1.0 mg/L, although exact performance requirements have not been fully defined.

The preanalytical and analytical sources of variations in hsCRP measurements have been reviewed.\(^18\) CRP has several advantages over other markers discussed to this point. First, the analyte is quite stable. Whole-blood samples are stable for 3 days at ambient temperature. Serum, plasma, and whole...
blood are stable when refrigerated for at least 7 days and for extended periods of time when frozen. Second, many automated hsCRP methods are commercially available (>20 in the United States). This plethora of methods greatly facilitates the widespread use of this marker in clinical laboratories. Third, an international secondary serum reference material (CRM 470, developed by the International Federation for Clinical Chemistry) is available for standardization of kit calibrators. The CRP concentration in CRM 470 is 39.2 mg/L.

Several studies have examined assay imprecision and method comparability of several commercial hsCRP assays. The imprecision of hsCRP methods at low CRP concentrations varies widely (Table), although a number of methods are capable of imprecision of <7% at CRP concentrations as low as 0.5 mg/L, a value that demarcates the 25th to 35th percentile in healthy populations. The standardization of the assays based on assignment to the appropriate quartile on the basis of a nephelometric comparison method is good for many methods, whereas other methods require additional standardization efforts (Figure). One of the challenges to standardization with CRM 470 is that the CRP concentration is 10 times higher than is appropriate for standardization of hsCRP. Development of a secondary standard reference material would benefit continued standardization efforts. The Centers for Disease Control and Prevention have initiated a project to standardize hsCRP assays. The first phase of this project is to identify a secondary reference material with characteristics that are the same as fresh patient samples. Results from this first phase indicate that it is feasible to use diluted CRM 470 as a reference material for hsCRP assays.

The second phase of the standardization project will involve working with diagnostic vendors to ensure accurate calibrator value assignment and to normalize assay results. When this project is completed, it should minimize assay inaccuracy and permit the use of hsCRP results generated by different assays in different laboratories to be used interchangeably with a common set of clinical decision thresholds. The College of American Pathologists introduced proficiency-testing material for hsCRP assays in 2002. This proficiency-testing program should aid in both the assessment of assay standardization and the performance of hsCRP methods in individual laboratories.

As mentioned above, the performance requirements for hsCRP assays are not clearly defined. The total analytical error for a given analyte typically can be up to 50% of the intraindividual biological variation. The total analytical error is equal to systematic error plus the random error. The systematic error is equivalent to the assay accuracy, and the random error equals 1.96 times the assay imprecision. Published intraindividual biological variability for CRP is 30% to 63%. On the basis of these 3 studies, the total analytical error is between 15% and 32%. Additional studies on intraindividual variation are necessary to further refine the estimate of true CRP biological variability. If the accuracy of hsCRP assays is ±10%, a goal that is achievable with a standardization program, then the allowable imprecision is 2.6% to 11% for a single hsCRP measurement. If multiple hsCRP measurements are made, then the allowable imprecision is greater. If 2 measurements are averaged, then the allowable imprecision of the assay becomes 3.7% to 15.6%. The overall imprecision of many current commercial hsCRP assays is <7%. This degree of imprecision may be adequate clinically, but this estimate remains to be refined and formally validated.

Conclusions

A number of atherosclerotic risk markers have been measured in research laboratories. On careful review of the status of these assays in terms of clinical outcome studies, analyte stability under ambient or refrigerated conditions, availability of multiple automated methods that are cleared by the US Food and Drug Administration for clinical use, adequate precision and accuracy to be clinically useful, and standardization, it was determined that hsCRP is the best available marker. Further research is required to better define the performance characteristics necessary for assays bearing the designation hsCRP. These characteristics include developing guidelines for total analytical error from a careful review of the intraindividual biological variability of the analyte under conditions that will be encountered in clinical practice, defining allowable random and systematic error limits based
Quartile agreement of hsCRP methods with samples from blood donors. The quartile of each sample was determined using the Dade BN II comparison method. The 25th, 50th, and 75th percentile cutoff concentrations for the Dade method were applied to the results from each of the other 8 methods, and the quartile assignments were compared with those obtained by the Dade method. Results of the Daiichi method with 67.8% quartile agreement are compared in A, results of the Denka method with 95.4% quartile agreement are compared in B, results of the DPC method with 93.8% quartile agreement are compared in C, results of the Iatron method with 94.6% quartile agreement are compared in D, results of the Kamiya method with 76.9% quartile agreement are compared in E, results of the Olympus method with 74.0% quartile agreement are compared in F, results of the Roche method with 76.2% quartile agreement are compared in G, and results of the Wako method with 91.7% quartile agreement are compared in H. Reprinted with permission from Roberts et al.21
on this information, validating these guidelines in the clinical setting, and completing the standardization efforts that are under way at the Centers for Disease Control and Prevention.

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