In this month’s issue of *Clinical Chemistry*, Anborgh et al. demonstrate that differences in assay configuration can produce major differences in the association of plasma osteopontin measurements with survival in prostate cancer (1.). Prediction of survival was found to be significantly better with assays that have a monoclonal antibody targeted to a specific epitope on the osteopontin molecule. This linkage between assay configuration and clinical utility may have major implications on the way reference materials and reference methods are formulated, because, as this report shows, the reference standards may need to focus on specific forms of the analyte.

Various national and international organizations are working to develop reference standards to help harmonize diagnostic assays. The European Union In Vitro Diagnostics Directive (98/79/EC) requires manufacturers marketing in the European Union to provide evidence that the values assigned to calibrators and control materials are traceable, through available reference measurement procedures and reference materials (when available), to standards of a higher metrological order (2, 3). The Joint Committee on Traceability in Laboratory Medicine (JCTLM) has helped guide the logistics of traceability with the use of European Committee for Standardization (CEN) and International Organization for Standardization (ISO) standards (4). ISO document 17511 distinguishes 2 broad categories of measurands: type A analytes, which are traceable to International System (SI) units, and type B analytes, which are not traceable to SI units (5). Type A analytes include a limited number of well-defined small molecules, such as electrolytes and steroid hormones, whereas type B analytes include most proteins and other laboratory measurements. Reference methods for type B analytes generally are less well developed, but JCTLM has a program for evaluating proposed methods.

Guidance on reference materials is provided in ISO document 15194. This document states, “The production and selection of reference materials is a multifactorial optimization and should be governed by clinical needs, production possibilities, metrological needs, analytical problems, economic, ethical and safety considerations, and the requirements needed for the end-use” (6). The choice of reference materials and reference methods is clearly a complex task, but the “requirements needed for end-use” should be an important part of this selection process. Therefore, if an assay is intended to predict a specific clinical outcome, the metabolic form of the analyte used for any reference method should be selected to optimize that particular use.

LC-MS has become a popular method for quantitating proteins. Smaller homogeneous analytes can be quantified directly by measurement of the intact molecule followed by amino acid analysis. For larger, less homogeneous proteins, however, the mass/charge ratios of peptide fragments generated by trypsin or other enzyme digestion may be used for quantification. When peptides are used, the selection of the “signature peptides” and the methods used for concentrating them before LC-MS measurement may substantially influence the correlation of the reference standard or reference method with the intended clinical use.

Two examples may help to illustrate these issues. The NIST has produced Standard Reference Material 2921 for the human cardiac troponin complex (7). The choice of the troponin complex was made with the assistance of IFCC and the Cardiac Troponin I Standardization Subcommittee of the AACC. Various recombinant and native troponin complexes were evaluated (8). The material chosen was purified from human heart tissue and consists of 3 subunits: troponin T, troponin I, and troponin C. NIST provided value assignments for each of the components. This material thereby serves as a reference standard for assay harmonization without explicitly providing data on which form of troponin is most useful for a given clinical application.
A second example is the recent publication by Fortin et al. in *Molecular & Cellular Proteomics* (9), which describes a procedure for quantifying small amounts of prostate-specific antigen (PSA) with LC-MS after protein depletion and trypsin digestion. Although the authors do not propose this procedure as a standard reference method at this time, their method is antibody independent and has good traceability. They selected a PSA peptide (LSEPAELTDAVK) that provides good LC-MS sensitivity and precision. PSA circulates in multiple forms, however, and the various immunoassays react differently with these forms and react with different parts of the PSA molecule. In addition, PSA has multiple potential clinical uses. The reference method and/or reference standard for early detection of aggressive prostate cancer may be different from those needed for early detection of less aggressive cancer. Likewise, the analytical form that predicts prostate cancer survival may be different from the forms that are optimal for early detection. The reference methods should be linked to the intended use, and the selection of the reference form of the analyte should be linked to clinical outcome data. Therefore, if the selected peptide is used as a reference for PSA quantification, it should be shown to have optimal utility for the selected clinical application.

The clinical utility of the osteopontin assay for predicting prostate cancer survival described in the report by Anborgh et al. is linked to monoclonal antibody mAb53 (1). This antibody exhibits maximal binding to the peptide sequence 165YGLRSK, which is a site for thrombin cleavage. The mAb53 antibody does not bind to the cleaved protein. Because osteopontin measurements made with assays that use this monoclonal antibody have stronger associations with survival in patients with prostate cancer, this peptide sequence appears to be a good “protein signature” and probably should be an important part of any reference method for osteopontin assays with this intended use. The authors also refer to the work of Christensen et al., who established that serine residues at positions 76, 78, and 81 in the osteopontin-binding site (75PSKSNESH) against which their second monoclonal antibody is directed are subject to phosphorylation in human milk and urine (10). The potential for such phosphorylation raises issues about the effects of posttranslational protein modifications and protein metabolism. These factors should also be considered when selecting peptides for reference measurements, because, as the authors suggest, the phosphorylation status may be health related.

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