The contribution of laboratory medicine to clinical cardiology has grown in importance over the years. Highly sensitive and specific biomarkers for the detection of myocardial damage, such as cardiac troponins, as well as assays for reliable markers of myocardial function, such as cardiac natriuretic peptides, are now available, assigning to the laboratory a pivotal role in the diagnosis and follow-up of patients with cardiac disease (1). This has been evidenced by the incorporation of these markers into new international guidelines (2–4). Among those, one of the most important documents outlines the recommendations for a new definition of myocardial infarction (MI), published by the joint European Society of Cardiology and American College of Cardiology committee (2). This consensus document has based the MI definition on biochemical grounds, a choice guided by the advent of cardiac troponins, biomarkers that provide higher sensitivity for smaller myocardial injury and virtually total specificity for cardiac damage (5). It is therefore important that these clinically relevant biomarkers, on which critical decisions will rest, are measured with highly reliable and standardized methods to achieve comparability of results, independent of the measurement test reagents and platforms as well as the laboratory where the procedure is carried out (6). Interchangeability of results over time and location would contribute considerably to improvements in healthcare because results of clinical studies undertaken in different locations or at different times could be universally applied. This would allow effective application of evidence-based medicine, as guidelines established by scientific or professional bodies often advocate use of specific decision limits for diagnosis and therapeutic intervention (3).

Because of an international patent, cardiac troponin T assay from only a single diagnostic manufacturer are commercially available, so that comparability of results for this marker is not a problem. Conversely, more than 15 different companies currently market assays for cardiac troponin I (cTnI) measurements; these assays use different standard materials and antibodies with different epitope specificities. As a consequence, analytical systems may give results that are unique to a particular method or instrument, so that different results from different cTnI assays and platforms may be obtained. Cooperative studies and external quality assessment schemes have shown quite large scatter. An IFCC-coordinated study of 14 platforms recently showed 20-fold differences in cTnI values among methods (7). The consequence inability to define common decision limits for cTnI may create a substantial problem for the clinical and laboratory communities when marker results are interpreted.

There is now an international agreement that standardization of quantitative measurements in laboratory medicine requires the consistent definition and application of a reference measurement system for calibration and validation of routine methods (8). Such a structure is based on the concepts of metrologic traceability and of a hierarchy of analytical measurement procedures. The importance of the metrologic principles has been described recently in an International Organization for Standardization (ISO) document, and the European directive on in vitro diagnostic devices follows this ISO standard by requesting its application for all in vitro diagnostic reagents used within the European Union (9). Key elements of the reference system are the reference measurement procedure and different kinds of reference materials. The reference procedure, which is calibrated with the primary reference material, is used to assign a certified value to a secondary reference material. Once the appropriate reference material is certified, this material and the manufacturer’s testing procedure can be used by industry to assign values to commercial calibrators. Clinical laboratories generally use routine procedures with validated calibrators, both from commercial sources, to measure human samples. In this way, the obtained value will be traceable to the reference procedure and materials, and measurement standardization, i.e., the process of realizing traceability and achieving trueness, will be reached. However, because no reference measurement procedure or appropriate reference materials for cTnI are available at present, manufacturers currently prepare their own calibrators and assign values on a mass basis of the chosen preparation, which is often not available to others, thus contributing to disagreement among assays (10).

The traceability model emphasizes in particular the importance of a detailed definition of the analyte to be measured. In certain cases, i.e., for analytes that are well-defined chemical entities, the definition of the analyte is straightforward. However, when considering much more complex substances, such as several protein biomarkers, including cTnI, the definition may not be as clear because of potential intrinsic or acquired heterogeneity. In biological samples, cTnI is present as a mixture of different molecular species. Intact cTnI and a spectrum of up to 11 modified products have been detected in sera from patients with MI (11). In turn, for definition of the analyte “cardiac troponin I”, it must be decided whether the term refers to (a) a mixture of different forms, i.e., free and complexed with troponins C and T, or to only one prevalent form; (b) composition classes (in terms of, for example, oxidation or phosphorylation); and (c) content classes (in terms of, for example, percentage of phosphorylation). As a matter of fact, the heterogeneity of cTnI may be circumvented by the definition of a unique, invariant part of the molecule that is common to all components of the mixture present in blood, e.g., the epitopes that are located in the central part of the cTnI molecule and are not affected by troponin IC or ITC complex formation and other “in vivo” modifications (12). Antibodies used for the development of cTnI assays should selectively recog-
nize these epitopes with a consequent increase in the homogeneity of immunoassay reactivity (13). Standardization of cTnI assays is in fact not possible if differences in antibody specificities among the assays are not minimized (14).

The major prerequisite for guaranteeing comparability of results among different assays, however, remains the availability of suitable reference materials. Several studies have shown that the quantitative differences in cTnI test values among currently available commercial assays are largely attributable to the lack of a common calibrator for use by the manufacturers of these assays. Katrukha et al. (15) showed that use as common calibrator of a material containing equimolar concentrations of human cardiac troponins I, T, and C significantly reduced the interassay variability of cTnI values for a positive troponin sample.

On the basis of this experimental evidence, an international agreement was reached recommending the selection as reference material for cTnI of a compound representing the natural and major form of the antigen in blood after tissue release, i.e., the troponin complex form (13). The AACC subcommittee for cTnI standardization, working in cooperation with the NIST, the IFCC Committee on Standardization of Markers of Cardiac Damage (C-SMCD), and diagnostic companies, has been involved in the identification of this type of material. The first phase of the process involved the evaluation of 10 different candidate materials, consisting of human native and recombinant protein, in liquid-frozen and lyophilized forms (10). Preliminary characterization studies using mass spectrometry to verify the material composition were conducted at NIST (16). Through 2 comparison studies conducted with the manufacturers of cTnI assays, the various preparations were evaluated and the best material was chosen. An extensive structural characterization of the selected material, a troponin ITC ternary complex purified from human cardiac tissue, was carried out at NIST by liquid chromatography coupled to mass spectrometry and tryptic digestion followed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The concentration value of cTnI in the material was certified through a combination of HPLC and amino acid analysis (17). The first method used reversed-phase HPLC to separate the 3 troponin subunits present in the reference material. cTnI quantification was based on the height of the cTnI peak and was interpolated from a calibration curve of peak height vs cTnI concentration derived from external calibrators prepared from purified human cTnI. The second method to determine the cTnI concentration in the reference material used amino acid analysis of the cTnI subunit purified from the troponin complex by reversed-phase HPLC, as described above. The reference material for amino acids, NIST SRM 2389, was used as external standard for the amino acid analysis. The measured concentrations of the amino acids alanine, valine, and leucine were used to calculate the concentration of cTnI, accounting for the number of each amino acid present in one cTnI molecule. The next phase of the program included an experimental evaluation of the ability of this reference material, used for direct calibration of commercially available methods, to achieve a significant improvement in comparability of results. The use of SRM 2921 as common calibrator in commercial systems did not, however, improve result comparability of cTnI measurements in human sera, indicating that the mere availability of a primary reference material is not sufficient by itself for standardizing cTnI results (18). There may be several underlying reasons. First consider that, although the content of the SRM 2921 material attempted to mimic the major form of cTnI found in biological specimens, the analytes in the reference material and in biological fluids are definitely nonidentical. Primary reference materials for heterogeneous proteins can serve only as surrogates for the analytes to be measured in patient samples. Although such materials resemble to some extent the analyte present in the human fluids, they may, however, represent only an “average” condition, and this may invalidate the basic rule of immunoprocesses: to compare “like with like”. Furthermore, it cannot be excluded that the purification procedures used can lead to partial modification of the structure of the troponin molecule, which in turn might affect the immunologic reaction, at least for some assays. NIST scientists have shown that some posttranslational modifications in the cTnI extracted from human heart may be attributable to the purification process (16).

An appropriate solution to these problems can be the identification of preparations with a composition and matrix similar to that in clinical samples and their use as secondary reference materials to calibrate commercial assays (19). In the case of cTnI, the selection of a panel of human serum samples appears to be the preferred option. The effects of the natural variation among donors can be minimized by use of pooled collections from numerous individuals. The cTnI concentrations of these samples could be assigned against the previously described primary reference material, thus assuring the traceability of patient results.

Several studies have shown that cTnI values compared more closely between the systems if one or more serum samples were used as the common calibrator rather than an artificial calibrator. In a study by Tate et al. (20), the harmonization effect was assessed by determining the among-systems CV after correction for calibration differences among 7 assay systems by reference to 2 different materials used as common calibrator, a purified ternary complex material and a serum sample collected from a patient with MI. Generally, test values were better harmonized among systems using the serum sample rather than the processed material as calibrator even if, in some samples, significant scatter among the different methods was still observed (20). In a similar study, performed by the AACC cTnI standardization committee, the alignment strategy using human serum pools produced a 5-fold improvement in intersystem variability. In particular, the variation (as the CV) among cTnI assay methods decreased from 82%-97% to 7%-28% (18).

Taken together, these works suggest that a small num-
umber of human serum pools (e.g., 5 samples containing cTnI around the clinically relevant concentrations) could serve as a secondary reference material for cTnI measurement. As has already happened for serum cortisol (21), these preparations should be selected and prepared under the auspices of the IFCC in cooperation with an internationally recognized provider of reference materials, e.g., NIST or the Institute for Reference Materials and Measurements (IRMM), according to recommendations from the relevant ISO document (22). However, use of human serum alone will not provide value assignment; therefore, a reference measurement procedure that can be reproduced within defined specifications is essential for certification of cTnI values in these secondary reference materials. Unfortunately, for cTnI, the search and the assessment of candidate reference materials have not been supported by the simultaneous development of a reference measurement procedure. This may have been the result of technical difficulties, but another reason might be that it was thought that a common reference material would be sufficient for reaching method standardization. A possible option is the development of a “high-level” immunologic procedure, based on the availability of monoclonal antibodies with well-defined specificities against epitopes of the cTnI molecule that can be considered pristine from the point of view of stereoechemical inhibition of the binding, calibrated with use of SRM 2921. The main argument against this option is that an immunologic procedure may be too dependent on a certain assay technology because this technique is an indirect measurement approach (23); however, alternative nonimmunologic techniques, such as mass spectrometry, that would otherwise be preferred for standardization lack the sensitivity for measurement of cTnI, which occurs in blood at very low concentrations. “Patient-like” secondary reference materials can also eliminate, by definition, the problem of noncommutability of reference materials with native samples (8). Commutability is defined as the ability of a reference or control material to show interassay properties similar to those of human samples. Only commutable materials can be used by industry for direct value assignment of manufacturers’ calibrators and trueness checks of laboratory tests, having great importance to ensure an unbroken traceability chain (24).

Because of this complicated situation, it is clear that progress in the standardization of cTnI immunoassays will be relatively slow and that some of the problems in this field will not be solved soon. As an interim solution, an assay harmonization approach that incorporates recalibration of various assays to give the same results has been advocated (25). In this case, a “designated” common comparison method should provide the basis for harmonization. Historically, a wild kind of harmonization already existed in which commercial companies, to fulfill US Food and Drug Administration demands, tried to adjust their assay results based on those of the first assay released in the market, i.e., the Baxter Stratus assay. Harmonization thus is possible only in a method-dependent manner. Furthermore, although methods can produce more or less similar results, these may be far from traceability and significantly biased in terms of trueness. Despite some practical problems, standardization of cTnI immunoassays should, therefore, be the goal whenever possible. In agreement with a metrologically correct approach, the IFCC C-SMCD has proposed that for the standardization and traceability of cTnI measurements, a reference measurement system is required (19, 26). This is to comprise a primary reference material; a matrixed secondary reference material, represented by a panel of appropriately selected and certified human serum samples; and a reference/consensus procedure for the value assignment of these secondary materials and evaluation of the analytical performance of field methods (Fig. 1). Working with this proposal in mind, it is essential that a uniform and rigorous outlook be maintained to ensure optimal utilization of all expertise available, including that in the diagnostics industry. I am sure that improvements will follow through both the advancement of knowledge and consensus on reasonable compromises.

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Fig. 1. Proposed reference (ref.) measurement system for cTnI.

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