The importance of analytical quality specifications for cardiac biomarker assays

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ABSTRACT

It is very important that cardiac biomarkers on which clinically relevant decisions will rest are measured with highly reliable assays. Adequate studies are needed before new methods can be implemented in the laboratory routine, and only well documented assays should be considered for clinical use. It is therefore critical that, as new biomarkers are proposed, quality specifications are developed. Only after appropriate analytical quality specifications are addressed, the issues pertaining to methodological differences that result in non-harmonized concentration values, and clinical interpretation of biomarker concentrations will be reconciled. Today, the technology to address many analytic problems is at hand, but commitment on the part of manufacturers and their customers in the laboratory and clinical communities is essential. The design control loop is not closed until the finished in vitro diagnostic system is adequately validated to meet the customer needs, including analytical quality specifications. It is essential to determine the attributes and performance characteristics of relevant competitive systems and their degree of acceptance by clinical laboratories in order to definitely demonstrate that user needs are met. The responsibility of defining and implementing these issues must be a shared responsibility among laboratorians, clinicians, industry, and regulatory agencies on an international front. To date, two sets of quality specifications have been published, one for cardiac troponin assays and one for B-type natriuretic peptide assays. Both address analytical factors, such as calibrator characterization, antibody specificity, assay sensitivity and imprecision, and interferents, as well as preanalytical factors, such as sample type and stability. It would be ideal if regulatory agencies, such as FDA in United States, accept these criteria for premarket approval clearance applications.

RIASSUNTO

L'importanza delle specificazioni di qualità analitica per le determinazioni dei biomarcatori

E' essenziale che le determinazioni dei biomarcatori cardiaci, sui cui risultati sono basate decisioni cliniche importanti, vengano effettuate con metodi altamente affidabili. Prima che nuovi metodi siano introdotti nella routine di laboratorio sono necessari studi adeguati e solo metodi ben documentati dovrebbero essere considerati per l'uso clinico. Di conseguenza lo sviluppo di specificazioni di qualità è una condizione critica ogni qualvolta viene proposto un nuovo biomarcatore. I problemi relativi alle differenze metodologiche capaci di generare disarmonie nei valori di concentrazione dei biomarcatori e nella loro interpretazione clinica potranno essere risolti solo dopo avere stabilito appropriate specificazioni di qualità. Oggi disponiamo della tecnologia per la soluzione di molti problemi analitici, ma è essenziale l'impegno da parte dell'industria e dei suoi clienti, nel laboratorio e nelle comunità cliniche. Il cerchio della progettazione del controllo può considerarsi chiuso solo quando il sistema diagnostico rifinito è validato adeguatamente, ai fini della sua corrispondenza alle necessità del cliente, incluse le specificazioni di qualità analitica. E' essenziale determinare gli attributi e le caratteristiche operative dei corrispondenti sistemi competitivi, nonché il loro livello di accettazione da parte dei laboratori clinici, per potere dimostrare definitivamente che le necessità dell'utilizzatore sono soddisfatte. La responsabilità di definire ed applicare questi punti deve esser condivisa dai laboratoristi, dai clinici, dall'industria e dagli organi regolatori a livello internazionale. Fino ad oggi sono state pubblicate due serie di specificazioni di qualità, rispettivamente per la determinazione della troponina cardiaca e per la determinazione del peptide natriuretico di tipo B. Entrambe prendono in considerazione fattori analitici, come la caratterizzazione del calibratore, la specificità dell'anticorpo, la sensibilità e la specificità del metodo, e le interferenze, come pure fattori preanalitici, come il tipo e la stabilità del campione. La situazione ideale sarebbe che le agenzie regolatorie, come l'FDA negli Stati Uniti, accettassero questi criteri per il rilascio dell'autorizzazione alla vendita.

BACKGROUND

It is important that cardiac biomarkers (CM) on which clinically relevant decisions will rest are measured with highly reliable and standardized methods. Theoretically, adequate studies are needed before new methods can be implemented in the laboratory routine, and only well documented assays should be considered for clinical use (1).

Usually, analytical validation studies of CM assays tend to be designed to allow an assay to pass minimum regulatory criteria. Often this means simply showing equivalent analytical (as well as clinical) performance to an

assay that already has, e.g., clearance by United States Food and Drug Administration (FDA). However, the studies used to establish assay characteristics often fail to adequately address the analytic and clinical needs of an assay to allow for consistent clinical interpretation across all patient subsets. These issues are highlighted when attempting to compare results of multiple assays that measure the same CM. It is therefore critical that, as new CM are discovered and proposed for clinical use, quality specifications for their measurement are developed. Only after appropriate analytical quality specifications are addressed, the issues pertaining to a) methodological differences that result in non-harmonized concentration values, and b) clinical interpretation of CM concentrations will be reconciled.

Today, the technology to address many analytic issues is at hand, but commitment on the part of manufacturers and their customers in the laboratory and clinical communities is essential. The design control loop is not closed until the finished in vitro diagnostic (IVD) system is adequately validated to meet the customer needs, including the laboratory requirements based in turn on medical needs. Regardless of how well an assay satisfies the requirements for medical utility, it is clear that a competitive marketplace drives manufacturers to develop products that may stand the competition. However, it remains essential to determine the attributes and performance characteristics of relevant competitive systems and their degree of acceptance by clinical laboratories guided by their physician-clients in order to definitely demonstrate that user needs are met. The responsibility of defining and implementing these issues must, therefore, be a shared responsibility among laboratorians, clinicians, manufacturers of commercial assays, and regulatory agencies on an international front. A major concern is that, frequently, there are large differences in the construction of each CM assay. In this respect, it is vital that all information on the assays is given. Although this appears to be a logical prerequisite, some manufacturers are quite quick in releasing assays without collecting and publishing these thorough data.

GOALS FOR QUALITY SPECIFICATIONS

The main objectives of quality specifications should be:

a. for manufacturers to endorse and then consistently follow the proposed recommendations;

b. to encourage that all package inserts for CM assays include uniform information on method design, pre-analytical and analytical performance characteristics, and clinical performance characteristics and that this information is published in peer-reviewed journals;

c. to encourage the design and the implementation of appropriate research projects that consider the quality specification issues as a priority; and

d. to encourage regulatory agencies to adopt a uniform set of criteria (specifically derived from quality specification issues) for manufacturers to provide when seeking clearance for new and/or improved assays.

To date, two sets of quality specifications have been published, one for cardiac troponin assays and one for B-type natriuretic peptide (BNP) assays (2, 3). Both address analytical factors, such as calibrator characterization, antibody specificity, assay sensitivity and imprecision, and interferents, as well as preanalytical factors, such as sample type and stability.

ISSUES TO BE ADDRESSED

Assay calibration

The first aspect that merits consideration is assay calibration. Lacking suitable international reference materials, for all CM assays manufacturers currently prepare and select their own calibration material, so that different purification procedures and type of calibration antigens [native or recombinant as, e.g., for BNP, free or complexed protein as, e.g., for cardiac troponin I (cTnI)] are employed. Together with different antibody specificities, this is the most important source of the disagreements among results from commercially available CM assays.

In order to determine the characteristics of the suitable calibrator materials to be used in commercial assays, definitive information about the synthesis and catabolism of CM is needed. As a matter of fact, the calibrator composition should closely resemble that of the molecule showing the greatest utility in a given clinical situation. For this type of definition, an evidence-based international agreement is required. For instance, the IFCC Commiitte on Standardization of Markers of Cardiac Damage has recommended calibration of cTnI assays against the material representing the natural and major form of the antigen in blood after tissue release, which is the complexed form (2). Katrukha et al. (4) showed that the 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.

For BNP-related peptides, with the exception of preproBNP, all the metabolically BNP-related molecules are likely to be present in plasma, which is the biological sample generally used for measurements (Figure 1). Because these peptides are so heterogeneous and their composition in human body fluids may vary significantly, calibrator materials can be surrogates only for the analytes to be measured in patient samples. Definitive evidence, obtained by studying the differential release characteristics of peptides in response to diverse physiologic and pathologic stimuli and their clearance and degradation mechanisms, is needed to determine which peptide fragments are present in the circulation. In addition, these peptides need to be measured to obtain the greatest clinical utility, which may vary depending on the clinical situation.

Antibody specificity

All the most important CM are proteins or polypeptides

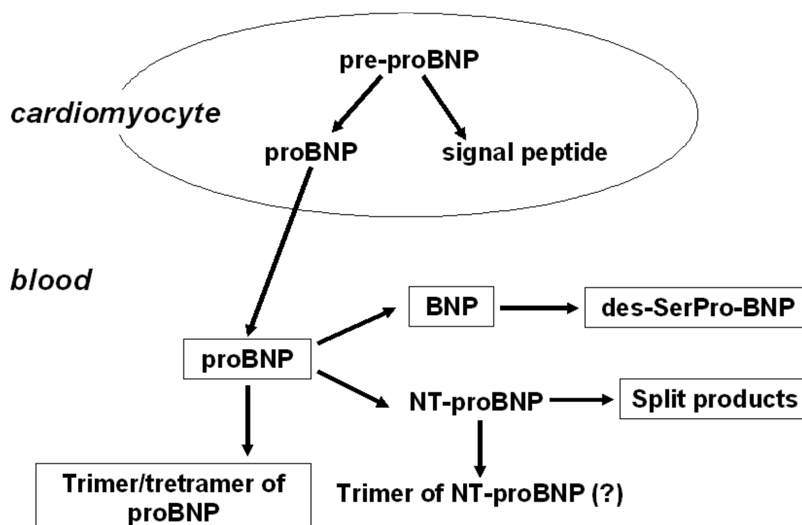

Figure 1
BNP-related analytes in blood. The presence in blood of the highlighted molecules has been demonstrated in experimental studies. Splitting of proBNP into BNP and NT-proBNP peptides occurring in the cardiomyocyte is not shown.

that are measured by a number of different immunoassays using specific antibodies directed to the respective antigens. In addition to the nature of the antigen employed for calibration, these immunoassays can, therefore, be influenced by the type and specificity of the antibodies present in the sandwich.

The issue of epitope location is important for cTnI assays because the amino (N)- and carboxyl (C)-terminal parts of the molecule are susceptible to proteolysis and this degradation may be related to the degree of tissue ischemia (5, 6). For this reason, antibodies used for the development of cTnI assays should selectively recognize epitopes that are located in the stable part of the molecule and are not affected by troponin complex formation and other in vivo modifications, with a consequent increase in the homogeneity of assay specificity (2, 7).

For BNP-related peptides, assays with critical epitope requirements may differ in their reactivity with circulating peptides, so that commercial assays, nominally measuring the same analyte, may be differently affected by cross reactivity problems (Figure 2). Plasma BNP may be overestimated if the sandwich against BNP is formed by an antibody directed against the ring structure of the molecule and the second antibody is directed to the C-terminal end. In this case, plasma intact proBNP is also detected by the assay (8). On the other hand, the combination of an antibody directed against the ring structure with an antibody against the N-terminal part of the molecule appears to be specific for the BNP, being, however, more prone to the peptide degradation by plasma proteases (9). This may result in a significant instability of the sample.

As regards amino-terminal-proBNP (NT-proBNP) measurement, the recognized antibody epitopes are also crucial for immunoassay specificity. It is likely, although

PolyAb against Nt end aa 1-10 (Biosite)

MoAb against aa 5-13 (Abbott)

MoAb against ring structure aa 14-21 (Scios)

MoAb against Ct end aa 27-31 (Shionogi)

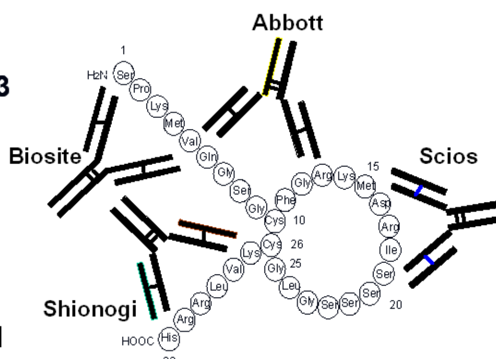

Figure 2
Epitope specificity of antibodies employed in commercially available BNP assays. PolyAb, polyclonal antibody; MoAb, monoclonal antibody; Nt, NH_2-terminus of BNP molecule; Ct, COOH-terminus of BNP molecule; aa, amino acid.

frequently not reported, that the antibodies used in many so-called "NT-proBNP" assays may also detect many of the circulating NT-proBNP split products, in addition to measure prohormone as well (10). Further, several clinical studies reported in literature have been performed by home-made non-commercial methods, which are not available to other research groups.

Different assays, theoretically measuring the same analyte, may thus produce significantly different results, not only in terms of proportional bias but also displaying significant intercept values in the comparison studies, meaning different analytical specificities (11, 12). For BNP measurements, no two assays are, therefore, analytically equivalent at present. Values are significantly dependent on the type of assay used, as a result of the specificity of the employed antibodies and of different calibration materials. As results are heavily method-dependent, it should clearly be stated that reference intervals and decision limits derived from clinical studies are only valid for the particular assay used and must not be extrapolated to other assays (13).

Assay sensitivity and imprecision

An undoubtedly important issue in the practical use of cardiac troponin measurements is the appropriate definition of assay sensitivity and imprecision. Accurate discrimination between "minor" myocardial injuries versus analytical noise requires assays that have low detection limit and high precision at low troponin concentrations. It has clearly been demonstrated that the percentage of patients recategorized from angina to myocardial infarction using the new diagnostic criteria based on troponin measurement may be critically dependent on the performance of the troponin assay used (14). The derived discordance in clinical classification is markedly important and has a significant impact on the prognostic evaluation of patients with suspected acute coronary syndrome (Figure 3) (15). Efforts to improve imprecision of cardiac troponin assays are, therefore, warranted: irrespectively of how the testing is performed (in the central laboratory or at the bedside), an optimal precision performance is needed at the low end of the assay measurement range. The demand for very precise cardiac troponin assays undoubtedly presents a difficult challenge, but results obtained with more recently released next-generation assays show that there has been substantial improvement in the precision and sensitivity offered by the newer assays (16). This type of low-end improvement is now considered by the manufacturers as the main goal in the design of new assays.

Assay interferences

Nonspecificity for CM assays can be the result of analytic interference. False test results may occur because of interferences with the antigen-antibody reaction in various immunoassays. Interferences from "heterophilic antibodies", which can mimic CM by linking the capture and detector antibodies, have been reported (17). These false results can lead to unwarranted and potentially dangerous procedures, e.g. cardiac catheterization, in the patient care. The lack of interference of heterophilic antibodies in an assay system should be carefully documented by measuring samples containing high concentrations of heterophilic antibodies in conjunction with treatment of the sample with agents to obviate their interference (2).

Icteric, lipemic, and hemolyzed samples might also be a problem in certain immunoassays. A thorough description of the experimental design that has been used to assess relevant interferences by high concentrations of the most important endogenous constituents is, therefore, required.

Sample type and stability

There are important issues related to the type of sample to be used for CM measurements and their in vitro

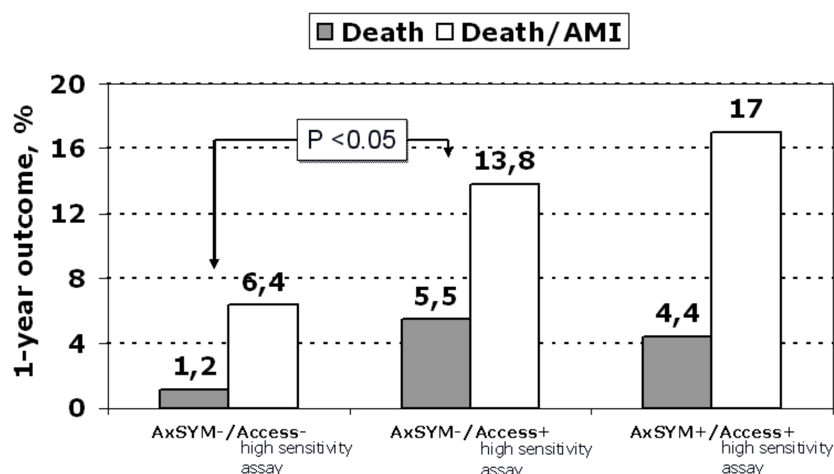

Figure 3
Frequency of clinical events in patients with unstable coronary artery disease in relation to negativity/positivity of troponin measured by two different assays (adapted from ref. 15). AMI, acute myocardial infarction.

Table 1
Cardiovascular biomarker measurement: issues to keep in mind when evaluating clinical studies

- Often the analytical characteristics of the assays are not adequately described (e.g., antibody specificity, optimal cutoff values)
- We need to know how the samples need to be collected and/or preserved for accurate measurements
- We need to know the stability of the samples over time (use of archived samples)
- Populations studied are often convenience populations for initial studies (need of confirmation in unselected populations)

stability. For BNP assays, the EDTA plasma is the only suitable specimen. Conversely, it appears that for measuring NT-proBNP by the Roche method, serum is the sample of choice. With this assay, EDTA plasma gave a consistent negative bias (8% on average) compared with matched serum samples, although studies did not indicate the variability among samples (18).

Also in troponin measurements, there can be significant difference between serum and plasma concentrations, at least for some analytical systems. Binding of heparin to cardiac troponins may reduce their immunoreactivity to various degrees, depending on the assay epitopes and the heparin concentration in sample tubes. On the other hand, the calcium chelator EDTA, splitting the calcium-dependent troponin complexes, may decrease the measured concentrations in troponin assays that preferentially measure these molecular forms. The use of anticoagulants for sample collection should, therefore, be studied and validated thoroughly before it can be recommended for practical use in the CM measurements (19).

Blood samples should not be collected in glass tubes when using an immunoassay employing in the sandwich an antibody against C-terminus for BNP measurement. It has been demonstrated that the above antibody is highly susceptible to the effect of kallikrein, a plasma protease activated by the contact with the wall of glass tube that degrades the C-terminal portion of BNP, making impossible the identification of the molecule by the immunoassay using the above reported antibody in the sandwich (20). The stability of the blood sample can therefore be obtained by the use of plastic collection tubes.

CONCLUSION

It appears that much work is needed to meet quality specifications in development and validation of CM assays. Concerns that have been addressed for cardiac troponins and cardiac natriuretic peptides will need to be addressed with the same scrutiny for all new proposed CM (21). To avoid the possibility for misinterpretation of a CM result for patient care, we have always to keep in mind that the performance characteristics of the employed assays should be adequately described (Table 1). Clinical shareholders that heavily rely upon CM in medical decision processes will substantially be impacted by the quality of assays in the marketplace.

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