Selection of Antibodies and Epitopes for Cardiac Troponin Immunoassays: Should We Revise Our Evidence-Based Beliefs?

Cardiac troponins are regarded as the most specific and sensitive of the currently available diagnostic techniques for detection of myocardial damage, and the redefined criteria used to classify acute coronary syndrome patients presenting with ischemic symptoms as myocardial infarction patients are heavily predicated on an increased concentration of these markers in blood *(1)*. Troponins also are the only markers identifying high-risk coronary patients who should be treated with antithrombotic agents, such as glycoprotein IIb/IIIa antagonists, and referred for invasive evaluation at the earliest opportunity. Not all troponin assays, however, are created equal; therefore, method selection by the clinical laboratory represents one of the major factors influencing the performance of this important biomarker for the diagnostic classification of an individual patient. Recent experimental data indicated that various commercially available methods have different limits of detection (and imprecision) for cardiac troponins *(2)*. Thus, the percentage of patients recategorized from angina to myocardial infarction based on troponin results as a diagnostic criterion is also critically dependent on the performance of the assay used *(3)*.

Formulation of assay reagents is also important in minimizing analytic interference. Falsely increased or decreased troponin results may occur because of interferences with the antigen–antibody reaction. Interferences from rheumatoid factors or human anti-mouse antibodies, which can mimic troponin by linking the capture and detector antibodies, have been reported *(4)*. Icteric and hemolyzed serum samples might also be a problem in certain immunoassays *(5)*. Finally, an interfering blood component inhibiting the binding of some antibodies against epitopes in the central part of the cardiac troponin I (cTnI) molecule has been described *(6, 7)*. The use of antibodies recognizing these epitopes, which are known to be less susceptible to proteolysis, has been recommended for the development of cTnI assays *(8)*, and many manufacturers have now endorsed this recommendation. Thus, the authors of studies on the negatively interfering factor predicted that many cTnI assays were likely to be affected by the factor. More and definitive experimental evidence was, however, required to permit identification and isolation of this component *(9)*.

The first of two studies by Eriksson et al. *(10)* published in this month's issue of *Clinical Chemistry* represents an important attempt to provide the necessary evidence about the factor that interferes in the troponin assay. Using an elegant experimental approach, the authors identified the factor present in the blood from one individual as a circulating troponin autoantibody. Furthermore, they found a correlation, in a small group of individuals, between the extent of inhibition and the troponin autoantibody titers estimated with a partially validated method. Some issues remain undefined, however, making the entity and the practical importance of the phenomenon unclear.

The use of serum samples from only one patient for isolating the interfering factor does not mean that the previously described, frequently occurring phenomenon is always and invariably attributable to circulating troponin autoantibodies. As correctly highlighted by the authors, the high frequency of occurrence of inhibiting factor is totally unexpected both on the basis of the previously reported prevalence of similar autoantibodies and from consideration of the results of the thousands of published analytical and clinical studies of cardiac troponin assays *(11)*. Only a single case of cTnI autoantibodies causing false-negative results has been reported previously in the peer-reviewed literature, and indeed, troponin testing would never have become the cornerstone for myocardial infarction diagnosis if there had been false-negative results in a large proportion of patients *(12)*. Furthermore, the coefficient of determination (r^2) obtained from the relationship between troponin autoantibody titers and recovery inhibition suggested that the titers explained only ~50% of the inhibition phenomenon, and some of the individuals in the "normal recovery" group also appeared to show detectable titers of inhibiting autoantibodies measured with the expressly developed assays [see Fig. 5 in Eriksson et al. *(10)*]. Other causes of the phenomenon therefore cannot be excluded *(9)*. Another problematic issue was the different saturation potencies of the autoantibodies presumed to be present in different individuals with low recovery of added troponin complex. Typically, the presence of inhibiting autoantibodies in serum leads to false-negative results, such as those described by Bohner et al. *(12)*: it is difficult to explain a commonly occurring partial inhibition.

To investigate the importance of their results, Eriksson et al. performed a second study *(13)*, in which they developed a cTnI assay that uses antibodies against epitopes in both the central and C-terminal parts of the molecule and thus was unaffected by the presence of troponin autoantibodies that bind the midmolecule region. Results from this assay were compared with results of two other commercial assays that incorporated only antibodies against the stable midfragment part of cTnI *(13)*. Unfortunately, many factors may influence the presented results and, at least partially, affect any conclusions. As already said, the sensitivities and imprecision of assays at low cTnI concentrations may significantly affect their clinical performance; consequently, it is quite expected to obtain better results with the new-generation assay. The assay has substantially improved precision, detection limit, and sensitivity compared with the corresponding first-generation Innotrac assay, which is based on the same "all-in-one" (Aio) analytical concept *(14, 15)*. On the other hand, when a more sensitive cTnI assay, the Beckman AccuTnI, was used for comparison, the differ-

ence in clinical performance was attenuated or even obliterated. Eriksson et al. *(13)* did not provide information on the time-dependent clinical sensitivity of AccuTnI, in comparison with the proposed new assay, for detecting myocardial infarction early after onset.

Other factors, such as the reactivities of the assays to various cTnI forms, i.e., the degree of equimolarity, and the possible influence of EDTA used as anticoagulant for sample collection in the first part of the study, may also be important contributors to the differences between the assays. Eriksson et al. *(13)* reported that the new assay produced a response to free cTnI that was ~40% lower than that of cTnI in binary complex with troponin C. Conversely, the same group showed that the antibodies selected for the first-generation Innotrac Aio assay reacted equally with free and complexed cTnI, and the same was shown for the Beckman assay by other authors *(16, 17)*. We recently reported that the time-dependent difference observed in the cTnI release kinetics after myocardial infarction can be explained by the different reactivities of the compared assays to various cTnI forms, differently distributed in the early vs late phases of myocardial infarction *(18)*. EDTA splits the calcium-dependent troponin complexes, thus decreasing concentrations measured by cTnI assays that preferentially measure these molecular forms *(19)*. A significant interference in the proposed assay can therefore be supposed, but information related to this aspect is lacking in the report. On the other hand, using the first-generation Aio assay, Hedberg et al. *(20)* showed that mean values for EDTA samples were 30% lower than those for matched heparin samples. Finally, as reported by Eriksson et al. *(13)*, cTnI stability with the new assay format could be an issue, mainly for the use of archived samples as happened in their study. It is conceivable that all of these factors can, at least in part, contribute to the observed differences between the assays.

In agreement with the conclusion of first study by Eriksson et al. *(10)*, clinical and laboratory communities should be aware that circulating troponin antibodies can interfere in cTnI assays. Before changing the current recommendations and alerting users and manufacturers to revise their scientific beliefs, however, additional specific and well-designed studies must be performed to clarify the practical advantages and the impact in the clinical setting of the proposed analytical changes.

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