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Editorials

Cardiac Troponins: Molecules of Many Surprises

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Monoclonal antibody–based immunoassays for measurement of cardiac troponins T and I, (cTnT and cTnI, respectively)[2] were first described approximately 25 years ago *(1, 2)*. Their promise was a more accurate diagnosis of acute myocardial infarction (AMI) due to improved tissue specificity. The advantages of cardiac specificity were realized soon after the clinical trials started, but an unexpected finding, increases of cTnT and cTnI that occurred "too early" in patients without traditional diagnostic criteria of AMI, was initially believed to compromise the biomarkers' diagnostic specificity. Further clinical studies established the real cause of these findings: minor myocardial damage. These new discoveries, made possible by cTn measurement, led to the redefinition of myocardial infarction *(3)*, and the eventual new clinical diagnostic category of acute coronary syndromes (ACS). In the new diagnostic paradigm, AMI is a subset of ACS, and both conditions can be diagnosed by cTnT and cTnI increases above predetermined cutpoint values. Further evolution of the diagnosis of ischemic myocardial injury has identified 7 types of AMI *(4)*, an unexpected development in the early years of troponin use.

Despite the proven cardiac tissue specificity of cTnI and improved specificity of the second generation cTnT assay, increases in the biomarkers' concentrations had been documented in nonischemic cardiac diseases such as renal or heart failure, after the administration of cardiotoxic agents, and in sepsis and stroke. Investigations into the causes of these increases revealed nonischemic myocyte necrosis as the cause of cTn release from the injured myocardium. It has also been established via the process called risk stratification that these increases, whether of ischemic or nonischemic nature, predict the possibility of worse clinical outcome even when cTn concentrations do not exceed the predetermined diagnostic cutpoint. In contrast to the diagnostic role of cTns, risk stratification is a statistical process that identifies a group of patients and assigns a certain probability of a clinical outcome based on cTn and other risk marker concentrations *(5)*.

When introduced into clinical practice, cTn measurement was expected to simplify diagnosis of AMI, but it appears that it has opened Pandora's box of clinical diagnoses. Although the cTns made diagnosing AMI somewhat simple by eliminating complications from nonspecific causes, the accurate interpretation of cTn concentrations still requires expertise from clinicians and from the laboratory professional alike. Further complicating this status quo are the numerous analytical variables that must be considered when an unexpected cTn result is encountered in clinical practice. Alternative splicing of the cTnT mRNA, various forms of posttranslational modification of both cTnT and cTnI, circulating monomers, dimers, or trimers of cTnT, cTnI, and troponin C (TnC), and physiological phosphorylation of cTnI have been reported in the literature as causes of discrepancy between cTn results and clinical presentation. Circulating anti-cTn autoantibodies, blocking assay antibody binding, and human antimouse antibodies (HAMA) may present additional analytical interferences with the troponin immunoassays and may lead to false-negative and false-positive troponin results.

The article by Katrukha et al. *(6)* in this issue of *Clinical Chemistry* investigates another form of posttranslational modification of cTnT, proteolytic degradation by thrombin, a serine protease coagulation factor that may be active at the site of ischemic tissue necrosis and is present in human serum following blood collection. The authors provide experimental evidence of cTnT proteolysis by thrombin that could be causing in vitro changes of the cTnT amino acid sequence. They demonstrated the shortening of the cTnT molecule in the presence of active thrombin that can be blocked by specific thrombin inhibitors. Using mass spectrometry, they identified the amino acid sequence of a 29-kDa proteolytic fragment and localized the cleavage site between amino acids 68 and 69, an arginine-serine sequence that is considered the specific site for thrombin activity. Their findings agree with a recent study by Streng et al. *(7)*, who presented preliminary evidence implicating thrombin as one of the potential agents responsible for cTnT degradation. Katrukha and coworkers have extended those studies and provided strong evidence that thrombin can cleave cTnT producing the observed degradation.

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[2] Nonstandard abbreviations: cTnT, cardiac troponin T; cTnI, cardiac troponin I; AMI, acute myocardial infarction; ACS, acute coronary syndromes; TnC, troponin C; HAMA, human antimouse antibodies.

Thrombin is not the only proteolytic enzyme that may be responsible for cTnT degradation. μ-Calpain and caspases play an active role in cTnT degradation as demonstrated by Di Lisa et al. *(8)* and others *(9)*. Although μ-calpain and the caspases are active primarily intracellularly and in vivo with physiological consequences, thrombin-mediated degradation appears to be extracellular and mostly in vitro. It may happen at the site of the infarction but it also takes place in vitro in the blood collection tubes during coagulation. The fact that multiple enzymes may work in parallel to digest cTnT can explain the fact that Katrukha and coworkers could not completely prevent cTnT degradation with thrombin inhibitors. Their Western blot analysis also showed the presence of smaller molecular weight cTnT fragments in patient sera but it is not clear if these fragments are from the further digestion of the 29-kDa thrombin-cleaved polypeptide or if they are produced from the intact cTnT molecule by other enzymes than thrombin. The investigation of the origin and the mechanism of production of these small fragments was not a goal of the current paper, but it may be worthwhile to study in the future.

Proteolytic digestion is not unique to cTnT. cTnI is also a target for proteolytic degradation both in vivo and in vitro *(9–11)*. Such identification of the causative agents and the resulting altered amino acid sequences of cTn supplies important information for laboratory scientists. Measurements of cTn concentrations are performed by immunoassays that depend on recognition of short amino acid sequences, so called epitopes, by the reagent antibodies. Altered or missing epitopes can diminish or prevent antibody attachment and lead to false negative laboratory results.

Although it is the job of the immunoassay manufacturers' to select antibodies that react with an epitope that is not subject to enzymatic degradation, the clinician using the assay result also must be aware that previously unrecognized epitope loss is a real possibility, and that apparent false negative results must be followed by alternative diagnostic procedures. The extensive posttranslational digestion of cTn will also complicate cTn assay standardization and harmonization as various antibody pairs will be affected differently by the degradation and the presence of the cleaved sequences. The investigation of enzymatic degradation of cTns may prove useful for clinical practice and when manufacturing blood collection tubes that contain enzyme inhibitors to prevent in vitro proteolysis of troponins.

With the recent introduction of mass spectrometry into the clinical laboratory, one may assume that immunoassay interference could soon be a problem of the past. However, mass spectrometric analysis may also require immunoaffinity extraction or immunoprecipitation by antibodies *(12, 13)* and cTn concentration measurement requires enzymatic digestion and the identification of specific polypeptide sequences by the mass spectrometer. Missing characteristic polypeptide sequences resulting from in vivo or in vitro proteolysis may prevent accurate measurement of cTn concentration even with mass spectrometry. Therefore, accurate quantification of cTn concentration requires the understanding of intra- and extracardiac proteolytic fragmentation of the cTn molecules.

This article in *Clinical Chemistry* is a reminder that cTn measurement is not a static science. The cTns have surprising characteristics and even after 25 years of active use they can still present new challenges to the clinical laboratory.

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