Thrombin Activation via Serum Preparation Is Not the Root Cause for Cardiac Troponin T Degradation

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To the Editor:

We read with interest the recent article by Katrukha et al. concerning thrombin-mediated degradation of human cardiac troponin T (cTnT) in serum *(1)*. In their study, Katrukha and coworkers confirmed the finding of Streng et al. that human coagulation factor II (thrombin) is a strong mediator of cTnT degradation *(2)*. They compared cTnT degradation in serum and heparin plasma collected simultaneously from the same patients with acute myocardial infarction (AMI), revealing substantial differences in cTnT fragment composition. They also performed in vitro experiments in which troponin T, thrombin, and hirudin were supplemented in various combinations. In addition, the cleavage site in cTnT was localized by use of mass spectrometry and probing of different proteolytic fragments with the use of various antibodies. Their main conclusion was that the primary 29-kDa cTnT fragment was mainly formed because of thrombin activation during serum preparation.

We believe that the evidence for thrombin as one of the main proteases for cTnT degradation is convincing. However, we disagree with their final conclusion because there is published evidence suggesting that cTnT degradation, including primary 29-kDa fragment formation, occurs in peripheral blood and is not solely caused by serum preparation.

First, Cardinaels and colleagues demonstrated that circulating cTnT undergoes degradation in serum following AMI, and the degradation pattern is time dependent after symptom onset *(3)*. They obtained serum samples from 18 patients with ST-segment elevation myocardial infarction at hospital admission and multiple standardized time points up to 72 h after admission. A clear time-dependent cTnT degradation pattern in serum was observed. Also, the presence of intact cTnT in serum was noticed in 3 of the 18 patients with AMI during the first 8 h after hospital admission. These data suggest that cTnT degradation takes place in vivo in a time-dependent manner following AMI.

Second, Katrukha et al. demonstrated a clear difference in primary cTnT fragment formation between serum and heparin plasma samples of patients with AMI *(1)*. Despite this finding, which the authors mainly focused on, Western blot images also displayed secondary cTnT fragments (16–20 kDa) in heparin plasma of patients with AMI, in addition to intact cTnT and its primary 29-kDa fragment. This is an interesting observation since previous data published by Mingels et al. demonstrated solely the presence of intact cTnT and its primary fragment when troponin standard reference material 2921 (NIST SRM 2921) was added in cardiac troponin-negative heparin plasma and incubated up to 72 h *(4)*. Hence, additional proteases might be involved in cTnT degradation following AMI.

These arguments indicate that cTnT fragmentation starts immediately after its release into the bloodstream and that the involvement of additional proteases in patients with AMI cannot be excluded. Moreover, it is highly likely that thrombin activation during serum preparation further enhances cTnT degradation to its primary fragment, though it is not the root cause. Also, the fact that secondary fragments are formed in heparin plasma of patients with AMI suggests that, in addition to thrombin, other serum proteases may be contributing to cTnT degradation following AMI. It is known that thrombin influences and balances its own generation and inhibition under physiological conditions *(5)*. However, during pathophysiological conditions, such as atherosclerotic plaque development and rupture, thrombin generation is enhanced while thrombin inhibitors are inactivated *(5)*. This imbalance results in proinflammatory effects of thrombin-mediated signaling. Thus, although cTnT-enriched normal human serum/plasma provides meaningful insights, it will remain crucial to perform further investigations in samples from patients with AMI.

Proper blood matrix selection will also be crucial in further investigations. cTnT degradation studies are thus far only conducted in serum and heparin plasma. It is known that thrombin generation is significantly increased during serum preparation, which, as Katrukha et al. and Streng et al. confirmed, has a preanalytic effect on cTnT degradation *(1, 2)*. Heparin plasma also has its limitations because the heparin–antithrombin complex does not completely inhibit thrombin activity. Also, additional thrombin generation is not prevented in this matrix. Therefore, additional research is required to identify a more proper matrix in which additional thrombin generation is prevented after blood withdrawal, while existing thrombin activity is fully inhibited.

In conclusion, we agree that thrombin activation due to serum preparation is an important contributor in degrading intact cTnT to its primary 29-kDa fragment. However, there are important indications that cTnT degradation in patients with AMI already takes place in the bloodstream and that additional proteases are involved in this process. We suggest further studies to be performed directly comparing serum

and multiple kinds of anticoagulated plasma in a time-dependent manner to investigate thrombin-mediated cTnT degradation more closely.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: D. de Boer, Central Diagnostic Laboratory, MUMC+.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.
Patents: None declared.

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Previously published online at
DOI: 10.1373/clinchem.2017.279182

In Reply

In the Letter to the Editor, Vroemen et al. disagree with the conclusion of our article that formation of the 29-kDa fragment of cardiac troponin T (cTnT)[1] observed in serum samples of patients with acute myocardial infarction (AMI) is mainly caused by the action of thrombin during sample preparation. In response, we have some clarifying comments.

In our studies, we have shown that thrombin cleaves cTnT at R68/S69 with the formation of the 29-kDa fragment. We and others *(1–3)* observed the same fragment in serum samples of patients with AMI together with 16- to 19-kDa fragments. We have also detected these fragments, although in a considerably smaller quantity, in heparin plasma samples of patients with AMI. The presence of the 29-kDa fragment in heparin plasma indicates that cTnT is cleaved in blood and/or necrotic myocardium at R68/S69, either by some yet unidentified protease(s), supposedly μ-calpain, which is capable of cleaving cTnT at the same site, or by thrombin, which might appear in blood as a result of thrombus formation during AMI. The 16- to 19-kDa fragments were identified as products of cTnT cleavage at R68/S69 from the N-terminus and at sites that lie presumably between residues 201 and 240 *(2)* at the C-terminus. This indicates that cTnT undergoes further degradation by protease(s) other than thrombin, as we have shown that R68/S69 is the only site of thrombin-mediated cleavage in cTnT. All these issues were noted in the Discussion section of our article. Meanwhile, the comparison of serum and heparin plasma samples simultaneously obtained from patients with AMI demonstrated that the full-sized cTnT abundantly present in heparin plasma was almost undetectable in serum, whereas the amount of the 29-kDa fragment in serum was dramatically increased (see Fig. 1 in our original article and Fig. 1 here). This convincingly confirms our findings that the formation of the 29-kDa fragment in serum samples is mainly caused by the action of thrombin, activated during serum sample preparation.

The work by Cardinaels et al. *(3)* cited in the letter by Vroemen et al. should be appraised by considering the thrombin-mediated degradation of cTnT in serum samples. Cardinaels et al. observed full-sized cTnT in 3 of 18 serum samples obtained early after AMI followed by stenting. We also observed the presence of the full-sized cTnT in several early serum samples; because they appeared to be different from most others, we thoroughly examined them. We discovered that these were not true serum samples but instead more closely resembled heparin plasma, as they contained intact fibrinogen and exerted anticoagulant activity (such samples were not included in our study). We attributed this discrepancy to the presence of heparin administered intravenously to patients before percutaneous intervention. Until now, we have observed only a negligible, if any, amount of full-sized cTnT in all true

[1] Nonstandard abbreviations: cTnT, human cardiac troponin T; AMI, acute myocardial infarction.