Peer Review File
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Reviewer Comments

Reviewer A
Comment 1: The authors have provided a state of the art analysis of current thinking relating to fragmentation and detection of cTnT and cTnI with respect to MI and non-cardiac or secondary cardiac conditions.

The narrative is in context. I do not have any comments that relate to alteration to the manuscript.

Reply 1: Thank you for the positive reception of our review.

Reviewer B
I enjoyed reading this review article. Proposal to further study troponin forms based on size to better classification for MI is indeed very thought provoking. I have a few minor comments:
Comment 1: Line 109: can you clarify more what you mean by detecting cTnT forms in blood with >60ng/L? does that mean you have to have at least 60ng/L of cTnT forms for detection? The way it is currently written is not clear.
Reply 1: Thank you for this comment, we have added "a minimal cTnT concentration of" to this sentence to clarify what we mean (Line 133).

Comment 2: Can you elaborate more for proposed explanations for why troponin is elevated in strenuous exercise? Is there any similar date in pregnancy?
Reply 2: As you suggested, we added the proposed mechanisms for cTnT elevations in vigorous exercise to the manuscript: "Several mechanisms have been proposed for cTnT elevations after vigorous exercise, i.e. physiologic remodelling of the myocardium, increased cardiac workload, or "bleb/vesicle" formation caused by transient ischemia" (lines 277-280). We also referred to systemic diseases, such as preeclampsia, that are also known for elevated troponin concentrations: "and similarly holds true for more systemic diseases like sepsis and pre-eclampsia" (line 270-271). However, it was our purpose to focus in our review especially on the circulating cTn forms currently found in some (non-)cardiac diseases in which cTn elevations are common. To the best of our knowledge, no research investigated yet cTnT forms in pregnancy.

Comment 3: On line 234, it is suggested that targeting large forms of cTnT will improved PPV for diagnosing acute MI, however earlier it was clarified that cTnT degradation is time dependent and large forms are found early in the course of MI while smaller fragments later in the course, so using larger forms of cTnT will miss late/delayed acute MI.
Reply 3: You are totally correct that if one would only test for large cTnT forms, one

might miss late/delayed acute MI. Unfortunately, for now it remains unclear what timeframe from symptom onset is reachable when detecting the larger cTnT forms only, this will all depend on the sensitivity of the future assay directed against those larger forms. Nevertheless, another way could be that such a new test rather acts as an additional simultaneously performed test to further specify the meaning of the cTnT elevations. We have now clarified this in the text (Line 294-296).

Comment 4: I think the association of strenuous exercise with the same cTnT sized fragments as that seen with ESRD deserves more discussion particularly because of the extensive literature associating elevated cTnT in dialysis patients with adverse CV outcomes.

Reply 4: We agree with the reviewer that the mechanism of troponin release is expected to be different between ESRD and strenuous exercise. Several mechanisms have been proposed to possibly explain release, however, to our opinion this is mostly hypothesis driven and unfortunately experimental data is very limited. We believe that more definitive insight in the mechanism(s) for cTn release and degradation in both vigorous exercise and ESRD is required before attempting to explain why both vigorous exercise and ESRD appear to have identical cTnT forms. We have added this to the manuscript (line 280-284).

Reviewer C

The authors did a great job providing a focused overview of the different circulating cTn forms and their potential clinical relevance. I have a few comments:

Comment 1: L74-76: "Though the hs-cTn immunoassay algorithms have excellent negative predictive values for acute MI rule-out (≥99%), their positive predictive values for acute MI rule-in remains suboptimal (75-80%)". It should be noted that in the US the PPV may be significantly lower due to the lower prevalence of MI patients in chest pain cohorts, for example see the following reference with a PPV of 56.6%: McCord J et al. Am Heart J. 2021 Mar;233:68-77. doi: 10.1016/j.ahj.2020.12.015. Epub 2020 Dec 26. PMID: 33373603.

Reply 1: Thank you for this insightful suggestion, we have added this comment to the manuscript (lines 86-88)

Comment 2: Section 2 "Analytical techniques to study circulating forms of cardiac troponin (cTn)". Can the authors please include the specific immunoassays they cite later in the manuscript, i.e., Giuliani et al. and Damen et al. (30, 31)?

Reply 2: Thank you for this suggestion, we have added a paragraph on immunoassays in general and on these specific immunoassays to our manuscript (line 165-185).

Comment 3: L128-129: "the detection limit for Western blotting is still relatively high: for cTnI this has not been described, for cTnT this is approximately 300 ng/L" Please

cite a reference or state that it is the authors' own experience.

Reply 3: Thank you for this comment. Indeed, the stated 300 ng/L was based on our experience and based on a study performed in our group. We have added this to the manuscript to further clarify (line 151-152).

Comment 4: L197-198: "But the question remains whether this is useful as cTnI does not seem to undergo time-dependent degradation." Please consider that for cTnTIC there may be a time-dependent degradation (into LMW-TIC and IC), as also shown by Vylegzhanina AV et al. (Clin Chem. 2019 Jul;65(7):882-892), and it may be of interest to compare the relative amount of cTnTIC complex in acute MI vs. other pathologies.

Reply 4: Thank you for this comment, we have added this to our manuscript (line 236-239).

Comment 5: Please also note JALM 2018 Nov;3(3):450-455 in which the authors suggest that the degree of proteolytic cTnI digestion increased with increasing severity of injury.

Reply 5: Thank you for this insightful suggestion, we have added this to our manuscript (line 240-242).

Comment 6: L212-214 and Figure 4 "Currently, all cTnT forms are detected by the fifth generation hs-cTnT immunoassay (Roche Diagnostics) since it targets two epitopes located in the stable middle region of the cTnT protein (Figure 4)". It is my understanding based on Vylegzhanina AV et al. (Clin Chem. 2019 Jul;65(7):882-892) that the Roche assay would not detect LMW-TIC.

Reply 6: This is a difficult question to answer. Vylegzhanina describes the presence of LMW-TIC in which the C-terminus is bound to the IC-complex and the core and N-terminus of cTnT are “removed” from the complex. It is indeed not possible to directly measure LMW-TIC using the current Roche assay. However, we expect that the Roche assay still can measure the “removed” core and N-terminus of cTnT (29kDa), because these antibodies bind to the core of cTnT. The concentration of LMW-TIC present in a blood sample would in theory be equal to the amount of 29kDa cTnT, which would indicate that the concentration of LMW-TIC is determined indirectly by measuring the concentration of 29kDa cTnT. However, this is of course dependent on the half-life of the various cTn forms. Therefore, we stated in our manuscript that all cTnT forms are detected by the fifth generation hs-cTnT immunoassay (Roche Diagnostics).

Comment 7: Figure 3. According to Vylegzhanina AV et al. (Clin Chem. 2019 Jul;65(7):882-892), there is no free full-size cTnT in MI, only full-size and 29 kDa cTnT as part of the cTnTIC complex and 25-27 kDa & 16-20 kDa central fragments.
Reply 7: Previous research from our group has shown that free intact 40kDa cTnT does exist. Using gel filtration chromatography (GFC), purified free intact 40kDa cTnT was

spiked in GFC buffer, lithium-heparin (LH) plasma and serum. All three matrices showed one main peak around 27-31 mL, indicating that, in our GFC set-up, free intact 40kDa cTnT is located there. A study by Damen et al. did both GFC and Western blotting of samples from 2 MI patients undergoing PCI. The GFC elution profiles showed a peak around 27-31 mL in all LH plasma samples in both patients, indicating the presence of free intact 40kDa cTnT. However, our current GFC set-up cannot differentiate between free intact 40kDa cTnT and the 29kDa cTnT fragment, therefore we also performed Western blotting of these samples. The presence of free intact 40kDa cTnT is most clear in patient 2, in which GFC elution profiles hardly showed any T-I-C complex peak, but still a free intact 40kDa cTnT band was found on the Western blot of de LH plasma samples (1).

Reference:

1. Damen SAJ, Vroemen WHM, Brouwer MA, Mezger STP, Suryapranata H, van Royen N, et al. Multi-Site Coronary Vein Sampling Study on Cardiac Troponin T Degradation in Non-ST-Segment-Elevation Myocardial Infarction: Toward a More Specific Cardiac Troponin T Assay. J Am Heart Assoc. 2019;8(14):e012602.