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SIBIOC DOCUMENT

Cardiac troponin variations in healthy and disease states: biochemical issues and role of renal function

Consensus Document of the Study Group on Cardiac Biomarkers of the Italian Societies of Laboratory Medicine: Italian Society of Clinical Biochemistry-Laboratory Medicine (SIBioC) and European Ligand Assay Society - Italy (ELAS)

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ABSTRACT

Since August 2018, the assay of cardiac troponin I (cTnI) and T (cTnT) with high sensitivity methods (hs-cTnI and hs-cTnT) is recommended by the Fourth Universal Definition of Myocardial Infarction as the specific biomarkers for the detection of myocardial injury. Even if the cTnI and cTnT assay is usually requested in clinical practice for the diagnosis of acute coronary syndrome (ACS) in patients admitted to emergence department (ED) with thoracic pain, this assay was demonstrated to be useful also for clinical evaluation of transient as well as chronic elevations of cTn levels in many disease states not clearly associated with myocardial ischemia. In the first part of this document, the latest acquisitions on analytical and biochemical issues related to measurement of circulating biomarker levels using hs-cTnI and hs-cTnT immunoassay methods will be taken into consideration. In the second part, the biochemical and pathophysiological mechanisms influencing the circulating cTnI and cTnT levels will be discussed in detail (in particular the pathophysiological distinction between reversible and irreversible myocardial injury). In the third part, some considerations related to clinical interpretation of hs-cTnI and hs-cTnT variations in healthy populations and patients with cardiac and non-cardiac diseases will be debated. In the final discussion, the Authors will comment the most important observations that can be derived

from the data reported in the literature, especially concerning the clinical role of hs-cTnI and hs-cTnT assay in patients with renal failure.

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Aims

Since August 2018, the assay of cardiac troponin I (cTnI) and T (cTnT) with high sensitivity methods (hs-cTnI and hs-cTnT) is recommended by the Fourth Universal Definition of Myocardial Infarction as the specific biomarkers for the detection of myocardial injury.[1] Even if the cTnI or cTnT assays are usually requested in clinical practice for the diagnosis of acute coronary syndrome (ACS) in patients admitted to emergence department (ED) with thoracic pain, these assays have been demonstrated to be useful also for clinical evaluation of transient as well as chronic elevations of cTn levels in many disease states not clearly associated with myocardial ischemia.[1-9] More specifically, increased hs-cTnI and hs-cTnT levels can be found in virtually all the acute or chronic clinical cardiac conditions, including: arrhythmias, cardiac ablation, cardiac contusion, infiltrative cardiac disorders, defibrillation shock, cardiac failure, and congenital heart diseases.[1-18]

However, elevated cardiac troponins have been commonly found in some patients with non-cardiac conditions, including: pulmonary embolism, impaired renal disease, sepsis/systemic inflammatory response syndrome and critically ill patient, rhabdomyolysis, burns, drug toxicity and stroke.[1-12] In particular, the fundamental role played by renal function on circulating cTn levels in patients with acute and chronic renal diseases should also be taken into consideration.[6, 7, 10-15] Finally, it is important to remember that even an intense physical exercise can cause an increase of hs-cTnI and hs-cTnT over the cut-off value, lasting up to 24-48 hours, in both healthy subjects or athletes.[6, 7, 9]

Taking into considerations the results of the most recent experimental and clinical studies as well as the recommendations of national and international guidelines, the Study Group of Cardiac Biomarkers of the Italian Societies of Laboratory Medicine SIBioC and ELAS decided to develop a document specifically dedicated to pathophysiological and clinical issues related to the variations of circulating levels of cardiac troponins in healthy individuals and patients. The document focuses in particular on two often underestimated aspects in international guidelines: the multiple circulating forms of cardiac troponins and the impact of renal function. These topics are addressed from both a biochemical and a clinical perspective, including their relevance in cardiovascular and non-cardiovascular contexts.

In the first part of the paper, the latest acquisitions on analytical and biochemical issues related to measurement of circulating biomarker levels using hs-cTnI and hs-cTnT immunoassay methods will be taken into consideration. In the second part, the pathophysiological mechanisms influencing the circulating cTn levels will be discussed in detail (in particular the pathophysiological distinction between reversible and irreversible myocardial injury). In the third part, some considerations related to clinical interpretation of hs-cTnI and hs-cTnT variations in healthy populations and patients with cardiac and non-cardiac diseases will be debated. In the final discussion part, Authors will comment the most important observations that can be derived from the data reported in the literature, especially concerning the clinical role of hs-cTnI and hs-cTnT assay in patients with renal failure.

Analytical and biochemical issues

Analytical issues

As with all the other immunoassays for peptides and proteins, it is essential to know the exact epitope location on

the amino acid peptide chain for an accurate pathophysiological and clinical interpretation of results obtained with the hs-cTnI and hs-cTnT immunometric methods.[8, 16-18] Unfortunately, the immunoassay methods, specific for cTnI or cTnT, employ a number of different antibodies directed to different epitopes located on the antigen molecule of the biomarker.[8, 9, 16, 17, 19] Moreover, protein chains of cTnI and TnT in cardiomyocytes and in circulation are combined with other proteins as well as in a large variety of different forms due the cleavage by proteolytic enzymes.[5, 16, 17] Consequently, different results from different methods may be obtained, and this problem may cloud interpretations of the reported data, inducing a relevant problem for the clinical and laboratory communities.[20-25]

In order to minimize these analytical problems related to the assays of cardiac troponins, since the year 2007, international guidelines[20] have recommended that the epitopes recognized by the antibodies must be specified by manufacturers, and that the antibodies specific for the more stable part of the cTnI and cTnT molecule should be preferentially used in immunoassay methods.

It is important to note that, at least currently, the hs-cTnT assay is offered worldwide by only one manufacturer, and so the hs-cTnT method shows less problems regarding the reproducibility of the measured biomarker concentrations. On the contrary, the commercial hs-cTnI methods usually used in the laboratory practice, differ one from another with regard to the type of the antigen used for calibration, the types of antibodies used and the epitopes to which they bind, and by the type of indicator molecule used.[2, 3, 8, 18-23] Moreover, for the detection of labeled antibody different technical approaches can be used, including: spectrophotometric, fluorescent, chemiluminescent, and electrochemical methods.[9, 18, 23] Indeed, numerous manufacturers have developed their own specific cTnI assays, leading to a situation in which cTnI measurements, using different methods on identical specimens, have been shown to differ by more than 20-fold.[18-23]

Unfortunately, despite the repeated attempts of International Federation of Clinical Chemistry (IFCC) Committee on Clinical Applications of Cardiac Bio-Markers to standardize the assays for hs-cTnI and hs-cTnT, at this moment, a significant heterogeneity still persists in antibody reactivities among the commercial assays, resulting in a large difference in the measured biomarker levels.[6-9, 18-26] Although several studies have clearly indicated that it is difficult (or even theoretically impossible) to completely harmonize immunoassays for hs-cTnI measurement, due to the use of monoclonal antibodies targeting different epitopes, other studies have demonstrated that it is possible at least to reduce the measurement bias among different hs-cTnI methods, by using recalibration procedures.[27, 28] As a consequence, currently, different hs-TnI assays do not produce equivalent results, and an "absolute" cTnI concentration cannot be obtained for hs-cTnI measured with different methods.[9, 20-28]

However, there are also some good news in the recent history of hs-cTnI and hs-cTnT immunoassays. In May 2018, the expert opinion from the Academy of American Association of Clinical Chemistry (AACC) and the IFCC Task Force[2] has stated that two criteria are needed for the definition of high-sensitivity methods for cTnI and cTnT) assays:

- the imprecision in measurement of hs-cTn concentration corresponding to the 99th percentile URL value should be ≤10%;
- measurable hs-cTn concentrations should be attainable at a value at or above the assay's LoD (Limit of Detection) in ≥50% of healthy individuals of both sexes.[2]

It is important to note that the Fourth Universal Definition of Myocardial Infarction[1] states that the detection of an elevated cTn value above the 99th percentile URL is defined as myocardial injury.

Considering the recommendations of these two international guidelines, all hs-cTnI and hs-cTnT methods should be characterized by important analytical and pathophysiological issues that unify all immunometric methods: for all hs-cTnI and hs-cTnT methods the imprecision at the cut-off value (*i.e.*, the 99th percentile URL) should be <10% CV. Indeed, several recent studies performed on the behalf of the Italian Study Group on Cardiac Biomarkers have clearly demonstrated that the hs-cTnI and hs-cTnT immunoassays show very similar imprecision profiles especially at the biomarker concentrations around the 99th percentile URL value,[6, 9, 23-25, 27-34] as reported in Table I and Figure 1. These data indicate that all imprecision profiles (Y axis) follow a reciprocal function. By applying this model, it can be observed that, on average, all methods measure a biomarker concentration of 6.1 ng/L with an imprecision of 10% CV. Considering that the 99th percentile ULR values of the hs-cTnI and hs-cTnT methods are usually higher than 10 ng/L,[26] the data reported in Table I and in Figure 1 demonstrate that all methods measure the cut-off value for the diagnosis of myocardial injury with a CV <10%,[6, 9, 23-25, 27-34] as recommended by all the national and international guidelines.[1-4, 7, 20]

Table I.—*Imprecision profiles of hs-cTnI methods.*

cTn level, ng/L	hs-cTnI Architect, CV%	hs-cTnI Access, CV%	hs-cTnI ADVIA, CV%	hs-cTnI Vitros, CV%	hs-cTnI Maglumi, CV%	hs-cTnT ECLIA, CV%
1	32.2	34.6	65.5	33.0	50.5	58.5
3	13.4	14.6	23.5	13.6	17.4	21.6
5	9.6	9.8	15.1	9.7	10.7	14.2
10	6.8	6.7	8.9	6.7	5.7	8.6
15	5.9	5.7	6.8	5.7	4.1	6.8
20	5.4	5.2	5.7	5.2	3.2	5,9
25	5.1	4.8	5.1	4.9	2.7	5.3
30	4.9	4.5	4.7	4.7	2.4	4.9
40	4.7	4.4	4.1	4.5	2.1	4.5
50	4.6	4.2	3.8	4.4	2.0	4.2

Imprecision profiles (expressed as CV%) of the most popular hs-cTnI and hs-cTnT methods used in the Italian clinical laboratories. These data were previously reported in some experimental and clinical studies supported by the Italian Study Group of Cardiac Biomarkers.[6, 9, 23-25, 27-34]
Methods and Manufacturers:
- Abbott ARCHITECT i-STAT® hs-cTnI assay (Abbott Laboratories Inc., Abbott Park, IL 60064, USA).
- Access hs-cTnI assay (Beckman Coulter Inc, 250 South Kraemer Blvd. Brea, CA 92821-6232, USA).
- ADVIA XPT hs-cTnI assay (Siemens Healthineers International AG, Hinterbergstrasse 14 6312, Steinhause, Switzerland).
- MAGLUMI® CLIA hs-cTnI assay (SNIBE Co, Ltd, National Biological Industry Park, Shenzhen, China);
- VITROS® High Sensitivity Troponin I Assay (Ortho Clinical Diagnostics, 001 US-202, Raritan, New Jersey, 08869, USA).
- Elecsys® Troponin T Gen 5 hs-cTnT (Roche Diagnostics International Ltd, Forrenstrasse 2, 6343 Rotkreuz, Switzerland).

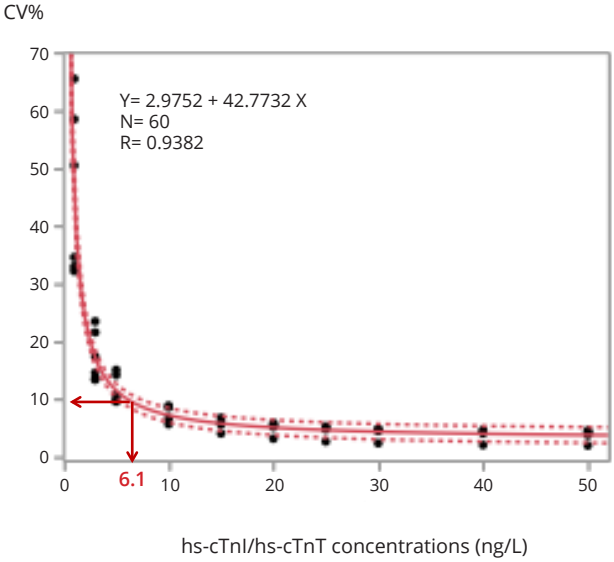

Figure 1.—Imprecision profiles of hs-cTnI and hs-cTnT methods. The figure illustrates the interpolation among the data of the imprecision profiles (Y-axis) of the five hs-cTnI methods and the hs-cTnT method, reported in the Table I. In the X-axis the concentration values of the cTnI (for the 5 methods) or cTnT (for the ECLIA method) are respectively reported. These data closely interpolate a reciprocal function, as illustrated in the figure. In the figure, the cTn value (X axis) corresponding to an imprecision equal to 10% CV is indicated by the red arrows (*i.e.*, 6.1 ng/L). The curvilinear fitting, using a reciprocal function, is calculated among the data (N.=60) related of imprecision profile of the hs-TnI and hs-cTnT methods, reported in Table I, using the JMP-17 statistical software (SAS, Statistical Discovery LLC, 920 SAS Camp Drive Cary, NC 27513).

Take-home messages

- The protein chains of cTnI and TnT in cardiomyocytes and circulation are bound with other proteins and exist in a large variety of different forms due the cleavage by proteolytic enzymes;[5, 16, 17]
- the immunoassay methods, specific for cTnI or cTnT, employ different antibodies targeting distinct epitopes located on the antigen molecule;[8, 9, 16, 17, 19]
- all the hs-cTnI and hs-cTnT methods exhibit significantly different analytical performances and cut-off values for the diagnosis of myocardial injury (*i.e.*, 99th percentile URL value);[6-9, 23-34]
- currently, different hs-cTnI and hs-cTnT methods yield varying results, which can complicate data interpretation and pose challenges for both clinical and laboratory communities;[20-25]
- nevertheless, all hs-cTnI and hs-cTnT methods demonstrate very similar imprecision profiles, particularly for biomarker concentrations near the 99th percentile URL value.[6, 9, 23-34]

Circulating forms of cTnI and cTnT

In the cardiomyocyte, cTnI and cTnT, together with troponin C (cTnC), form a structural unit and a functional complex in the sarcomere, playing a fundamental physiological role in the contraction of myocytes, both at rest and during exercise.[5-9, 17, 18, 21-24, 35-41] The total content of

cTnI and cTnT in the myocyte, is predominantly present in the sarcomere (more than 90%), while the remainder (<10% of the total) is mainly found in the cytoplasm.[35-41] In addition, cTnI and cTnT present in the cytoplasm are primarily contained in vesicles and have a structure different from that found in the sarcomere.[35-41] Indeed, the cytoplasmic troponins have a molecular weight lower than the sarcomeric ones (*i.e.*, 15 to 29 kDa for cTnI *vs.* 22.5 kDa to 39.7 kDa for cTnT).[35-41] It is currently believed that cardiac troponins can transit in the cytoplasm, where the protein chains are degraded with subsequent reuse of the amino acids to form new proteins.[16-23, 35-40]

In a systematic review published in 2021, Chaulin AM[17] suggested that the degradation and elimination of cTnI and cTnT molecules from the blood occur through three different ways, which may be closely related to each other:

- capture of cTnI and cTnT by cells of the reticuloendothelial system and intracellular cleavage;
- biomarker cleavage by proteolytic enzymes directly in the bloodstream;
- elimination of cTnI and cTnT molecules through the blood-tissue filters/barriers (glomerular and blood-salivary) into other biological fluids (in particular urine).

It is usually reported that, in patients with acute myocardial infarction (AMI), the peak values of hs-cTnI and hs-cTnT occur from 10 to 24 hours after onset of symptoms and remain elevated for 4 to 10 days.[1-9, 16-23, 36-40] However, the pattern and the magnitude of cTn elevations are highly variable among patients because the size of the AMI and the rapidity of cTn washout are influenced by reperfusion.[42-48] Accordingly, different forms and fragments of both cardiac troponins have been identified in the circulation, originating from intracellular or extracellular processes, and these different molecular forms complicate the immunometric determination and the pathophysiological interpretation of the circulating levels of cTns and their variations in both healthy subjects and patients with cardiovascular diseases.[16-23, 36-48]

Despite numerous studies reported in the literature, the fragmentation process of cardiac troponins *in vivo* is not completely understood, because it may depend on degradation, phosphorylation, ubiquitination, complex formation, and binding to specific anti-cTn immunoglobulins.[16-19, 21-23, 36-40] In patients with AMI, cTnT has predominantly been found intact (37 kDa) in the first hours after presentation, and afterwards as primary (29 kDa) and secondary fragments (15–20 kDa).[37, 38] In particular, the primary fragment is cleaved at the N-terminal end, whereas the secondary fragments are further cleaved at the C-terminal end.[14, 37, 38, 41-44] Only few studies described the degradation of cTnI in circulation of AMI patients, and usually these studies were carried out with not high-sensitivity methods.[16-18, 21-23, 45, 47, 48]

The results of a recent study have suggested that the half-life elimination of cTnI and cTnT was from 5 to 16 hours shorter than previously reported in patients with AMI.[44] The authors adopted an innovative experimental design for cTnI and cTnT kinetics study in humans. Patients with AMI (N.=20, mean age 64.5 [SD: 8.2] years; 4 women) were included in the study within 24 hours after revascularization and underwent plasmapheresis to obtain plasma with a high cTn concentration.[44] After at least 3 weeks, patients returned for an autologous plasma re-transfusion followed by blood sampling for 8 hours. Plasma cTn concentrations were measured with four different hs-cTnI methods and the ECLIA hs-cTnT assay. The median clearance of cTnI ranged from 40.3 mL/min (95% CI, 32.0-44.9) to 52.7 mL/min (95% CI, 42.2-57.8). The clearance of cTnT was 77.0 mL/min (95% CI, 45.2-95.0).[44] The authors concluded that. according to the results of the study, a considerably longer duration of cardiomyocyte cTn release after AMI than previously thought, was observed.[44]

Recent studies indicate that patients with end-stage renal disease had only small cTnT fragments with molecular weights comparable to the secondary fragments as reported in AMI patients.[49] It is not clear if these cTnT fragments observed in patients with end-stage renal disease may be comparable with those of AMI patients or whether they represent different types of disease-specific fragments.[49] Moreover, some experimental studies on rats indicated that both liver and kidney can mediate the clearance of cardiac troponins.[50]

Although large studies evaluated the cross-sectional association between Estimated Glomerular Filtration Rate (eGFR) and hs-cTns, there is a lack of prospective, longitudinal studies in stable chronic kidney disease (CKD) patients, monitoring over the time the interaction between change in hs-cTn concentrations and eGFR. In particular, Chesnaye *et al.*[51] reported that in CKD patients (N.=171, M/F=0.67, age ≥65 years, mean age 75 years) hs-cTnT increases over time as renal function decreases. Moreover, lower CKD stage (<15 mL/min/1.73 m^2) was independently associated with a steeper hs-cTnT increase over time as demonstrated for other well established cardiovascular risk factors.[51] Accordingly, Chesnaye *et al.*[51] concluded that hs-cTnT values increase independently over time in CKD patients with lower eGFR, suggesting

that the renal impairment may contribute by itself to increase the cardiac stress and produce myocardial injury.[51]

Smaller biomarker fragments have been measured using hs-cTnI and hs-cTnT methods also in healthy adult subjects after intense and prolonged physical exertion (such as a marathon or bicycle race).[6, 7, 9, 18, 23, 41-43, 52-54] Indeed, recent studies showed that after intense and prolonged physical exertion, some forms of the biomarkers, with reduced molecular weight (MW) and faster plasma clearance than sarcomere-bound troponins, can be measured with hs methods.[7, 9, 18, 23, 41-43, 52-54] However, even the most recently developed methods are not able to identify and accurately measure these circulating forms with lower MW without the use of preliminary and complex chromatographic procedures performed before the immunometric assay of the biomarkers.[7, 9, 41-43, 52-54]

Take-home messages

- More than 90% of the total content of cTnI and cTnT in the myocyte is localized in the sarcomere, while the remaining (<10% of the total) is mainly found in the cytoplasm;[35-41]
- the cytoplasmic troponins have a molecular weight lower than the sarcomeric ones (*i.e.*, 15 to 29 kDa for cTnI *vs* 22.5 kDa to 39.7 kDa for cTnT);[35-41]
- the *in vivo* fragmentation process of cardiac troponins is not fully understood; it may be influenced by degradation, phosphorylation, ubiquitination, complex formation, and binding to specific anti-cTn immunoglobulins;[16-19, 21-23, 36-40]
- in patients with AMI, peak levels of hs-cTnI and hs-cTnT typically occur between 10 and 24 hours after symptom onset and remain elevated for 4 to 10 days.[1-9, 16-23, 36-40] However, a recent study suggested that the elimination half-life of cTnI and cTnT may be 5 to 16 hours shorter than previously reported ted;[44]
- in patients with end-stage renal disease[49] and in healthy adults after intense and prolonged physical exertion (*e.g.*, marathon running or cycling), circulating forms of hs-cTnI and hs-cTnT with lower molecular weight (MW) have been detected;[6, 7, 9, 18, 23, 41-43, 52, 53]
- notably, even the most advanced hs-cTnI and hs-cTnT assays cannot accurately measure these lower MW circulating forms without employing complex preliminary chromatographic techniques.[41-43, 52-54]

Pathophysiological mechanisms

Cardiac troponin levels and cardiomyocyte renewal

In 2017, the Consensus Statement on the Cardiomyocyte Regeneration stated that the term "cardiomyocyte renewal" is defined as the ability to replace lost cardiomyocytes by new ones.[55] The cardiomyocyte renewal was evaluated in experimental animals and humas using different complex and sophisticated techniques, such as evaluation of either apoptotic or mitotic markers, analysis of thymidine analogs in cardiomyocyte dividing cells, or measuring incorporation of nuclear bomb test-derived ^{14}C into genomic DNA.[55-64]

Overall, all the studies on cardiomyocyte renewal indicate that cardiomyocyte exchange is highest in early childhood and decreases gradually throughout life to <1% per year in adulthood, with similar turnover rates in the major subdivisions of the myocardium.[55-64] In particular, these studies indicate that at the age of 25 years, approximately 1% of cardiomyocytes turn over annually, and the turnover rate decreases to 0.45% at the age of 75 years resulting in about 40-50% of the ventricular cardiomyocytes being exchanged during life.[56-58] Moreover, growth of the heart during embryonic and fetal development involves an absolute increase in the number of cardiomyocytes and is brought about by differentiation of precursor cells and by division of relatively immature cardiomyocytes.[55, 58] In healthy adults subjects, the cardiomyocyte renewal depends on the myocardial mass, and so it is higher in men compared to women.[58] Another important observation is that cardiomyocyte renewal rates may be higher after cardiac injury than under normal conditions.[55, 60, 61] In particular, some studies on heart regeneration in both neonatal and adult rodents found an increase in cardiomyocyte proliferation directly after a myocardial lesion.[65, 66]

A recently published paper[67] studied the cardiomyocyte renewal in 52 patients with advanced heart failure, 28 of whom received Left Ventricular Assist Device (LVAD). The authors measured the concentration of nuclear bomb test-derived ^{14}C in cardiomyocyte genomic DNA and performed mathematical modeling to establish cardiomyocyte renewal in heart failure with and without LVAD unloading.[67] The results of this study show that cardiomyocyte generation is minimal in end-stage heart failure patients compared with the healthy heart. However, patients receiving LVAD therapy, who showed significant functional and structural cardiac improvement, had a >6-fold increase in cardiomyocyte renewal relatively to the healthy heart.[67] These data suggest that the human heart present a poten-

tial cardiomyocyte regeneration in human heart disease, which could be exploited therapeutically.[67]

Some Authors have recently suggested that the circulating levels of hs-cTnI and hs-cTnT measured in healthy adult subjects may be considered a reliable index of the physiological cardiomyocyte renewal of human myocardium tissue.[6, 7, 9, 18] However, it is very difficult to demonstrate a close relationship between cardiac troponins circulating levels and myocardial renewal, because even the most sensitive cardiac imaging techniques, such as cardiac Positron Emission Tomography (PET), may not be able to accurately measure *in vivo* the low variations in myocardial renewal,[6, 7, 54, 58] due to an inadequate spatial resolution.[68] Despite the experimental difficulties in measuring the *in vivo* myocardial renewal in healthy individuals and in patients with cardiovascular diseases, some data are currently available supporting the hypothesis of a close relationship between cardiomyocyte renewal and hs-cTns circulating levels.[6, 9, 18, 69]

The mean biomarker concentration in healthy adult subjects is relatively low, close to the LoD of hs-cTnI and hs-cTnT methods (*i.e*., usually <4 ng/L). Moreover, the circulating biomarker levels in healthy subjects are relatively stable as demonstrated by several studies designed for the *in vivo* evaluation of cTnI and cTnT variations in healthy subjects and in patients with cardiovascular diseases.[6, 69-71] In 2021, Diaz-Garzon *et al.*[70] reported the results of a meta-analysis concerning the cTnI and cTnT variations including different sampling intervals and states of health. This meta-analysis included 16 studies on cTnI (11 on hs-cTnI) and 15 on hs-cTnT methods. The results of this study demonstrated that both cTnI and cTnI circulating levels are relatively stable in healthy subjects and that the estimates related to diseased subjects were similar to those found in healthy individuals for all settings.[70]

More recently, another meta-analysis was performed taking into consideration only data on biomarker biological variations found in adult healthy subjects[6] using hs-cTnI and hs-cTnT methods.[6] This meta-analysis,[6] including 10 studies for hs-cTnI and 4 for hs-cTnT, demonstrated that biomarker levels are stable if evaluated over different periods of time in healthy subjects (*i.e*., from 1 hour to 9 months). In particular, the individuality index of biological variability of hs-cTnI and hs-cTnT presents value similar to that of creatinine (on average about 0.3),[72] which is a biomarker closely related to the skeletal muscle mass renewal.

In 2022, Koerbin *et al.*[71] used a large cTnI data bank to evaluate the intra-individual biological variability (CVi %) of cTnI admitted to the Emergency Department (ED) patients without AMI. These Authors evaluated the effects of gender, age, climatic season, and time between samples to assess whether CVi changed between the individuals and different clinical conditions using ARCHITECT hs-cTnI method (Abbott ARCHITECT STAT high sensitive troponin-I [package insert SP25-37]).[71] The results of this study[71] indicate an intra-individual index of biological variability <0.20 for all conditions studied. It is important to underline that an intra-individual index <0.6 is generally considered to have a good correlation with specific individual physiological characteristics (such as sex, age and body mass).[6, 69, 72] Accordingly, a biomarker characterized by individual index <0.6 (such as cardiac troponins and creatinine) may show significantly biological variations over time that could remain within the reference intervals estimated from the general population;[6, 69, 72] in these cases the adoption of reference intervals to evaluate the specific result is unsuitable.

Even the variations according to age suggest a close relationship between cardiac renewal and circulating biomarker levels of hs-cTnI and hs-cTnT: in fact, the highest values of hs-cTnI and hs-cTnT are observed in the first month of life, followed by a rapid fall during the first six months, and a slower decline during childhood that finally reach a plateau during adolescence.[73-80] In general, the lowest sex-specific differences have been observed in pubertal ages, with higher concentrations in boys, showing a more marked and progressive increase from 14 to 18 years.[80, 81] Healthy adult subjects (>20 years) show a plateau in the hs-cTnI and hs-cTnT levels until 55 years with a progressive rise of the biomarker concentrations during the senescence, which is earlier and more evident in men compared to women at the same age. After 80 years of age, the difference in biomarker circulating levels between males and females tends to reduce progressively. The results of these studies underscore the importance of interpreting hs-cTnI and hs-cTnT concentrations according to age and sex.[69-82]

Another relevant point under discussion is that both cardiomyocyte renewal and circulating hs-cTnI and hs-cTnT levels are differently regulated and affected by sex hormones.[6, 7, 9, 24, 69-71] The higher hs-cTnI and hs-cTnT levels in age-matched males compared to females during all ages of life, are related to the myocardial mass and to the cardiac renewal. Indeed, *in vivo* ultrasound and magnetic resonance studies, as well as studies in Anatomy and Forensic Medicine, have demonstrated a greater myocardial mass in males compared to females,[83, 84] considering that the weight of the heart is on average 300 g (range 280-340 g) in men and 250 g (range 230-280 g) in women.[85]

Several studies performed in transgender subjects confirm the pathophysiological hypothesis that androgenic steroid hormones (particularly testosterone) are generally associated with higher hs-cTnI levels.[86-92] Moreover, higher levels of testosterone are also associated to an increased cardiovascular risk in transgender persons:[86-92] in fact, some transgender subjects assume androgenic steroid hormone therapy (particularly testosterone) in order to help align their physical traits and gender identity.[86-92]

Considering the most recent evidences, one might assume that the endogenous testosterone contributes to the increase not only to the muscular skeletal and cardiac mass, but also to cardiac troponins circulating levels in healthy males, contributing to the increase in cardiovascular risk in men compared to the women at the same age.[6, 7, 69, 79, 82, 86, 88, 93-106] The results of several recent studies, clearly demonstrated that the cardiovascular risk is significantly higher in apparently healthy men than in healthy women with concentrations of hs-cTnI and hs-cTnT in the upper tertile of the biomarker distribution.[6, 7, 9, 93-106] Therefore, these results also indicated that the measurement of hs-cTnI and hs-cTnT circulating levels may be useful to early identify, in the general population, the asymptomatic individuals at higher risk of progressing to symptomatic heart failure or developing Major Adverse Cardiovascular Events (MACE) over ≥6 months, such as patients aged >55 years and with comorbidities.[6, 7, 9, 93-105]

In conclusion, several experimental and clinical data strongly indicate that there is a close relationship between circulating hs-cTnI and hs-cTnT levels and cardiovascular risk, which in turn are also significantly related to age and sex.[55-59, 61, 62, 67, 69, 71, 93, 95, 106]

Take-home messages

- Studies on healthy humans indicate that cardiomyocyte renewal is highest in early childhood; while, at the age of 25 years approximately 1% of cardiomyocytes only turn over annually, and the turnover rate decreases to 0.45% at the age of 75 years.[55-58] Therefore, about 40-50% of the ventricular cardiomyocytes are exchanged during the life;[56-58]
- cardiomyocyte renewal rates may increase in patients with cardiac injury compared to normal physiological conditions;[55, 60, 61, 65-67]
- some authors have recently proposed that circulating levels of hs-cTnI and cTnT in healthy adults could serve as a reliable marker of physiological cardiomyocyte renewal in human myocardial tissue;[6, 7, 9, 18]
- the hypothesis of a close relationship between cardiomyocyte renewal and cardiac troponin turnover rates is supported by some experimental and clinical evidence demonstrating shared and interrelated pathophysiological features,[6, 7, 9, 18, 55-58, 60, 61, 65-67] including:
  - low and stable values of turnover rates;
  - parallel variations related to age (*i.e.*, from neonatal period to senescence);
  - close associations with cardiac mass and sex;
  - increased values after myocardial injury;
  - numerous experimental and clinical data strongly support a link between circulating hs-cTnI and hs-cTnT levels and cardiovascular risk, which is also significantly influenced by age and sex.[55-59, 61, 62, 67, 69, 71, 93, 95, 106]

Reversible *versus* irreversible myocardial damage

Several pathophysiological mechanisms can produce either reversible or irreversible damage on cardiomyocytes, depending from the intensity and the duration of injury.[1, 36-40, 43] It is commonly assumed that a reversible damage is quickly repaired by the cardiomyocyte, which immediately afterwards, resume its normal activity; on the contrary, an irreversible damage produces the death of the cardiomyocytes.[36-40, 43]

During an injury causing only reversible damage, the lesion regarding the plasma membrane of the myocardial cell may produce only a transient rupture of the cardiomyocyte membrane, thus inducing the leakage of a limited amount of cytoplasm containing complexes or microparticles including mainly cTnI and cTnT molecules in degraded form with low MW.[36-40, 43] Sometimes, a stress factor may produce an extrusion from the cardiomyocytes of some vesicles containing degraded troponins molecules, for example in the form of circulating exosomes or membrane protrusions (blebs)[36-40, 43] (Figure 2).

Death of cardiomyocytes can occur by means of different programmed and non-programmed (*i.e.*, necrosis) mechanisms.[36, 39, 40, 55-58, 61, 107-114] In particular, it was suggested that the programmed death of cardiomyocytes can be produced by several different mechanisms, such as: apoptosis, autophagy, necroptosis, pyroptosis, and fer-

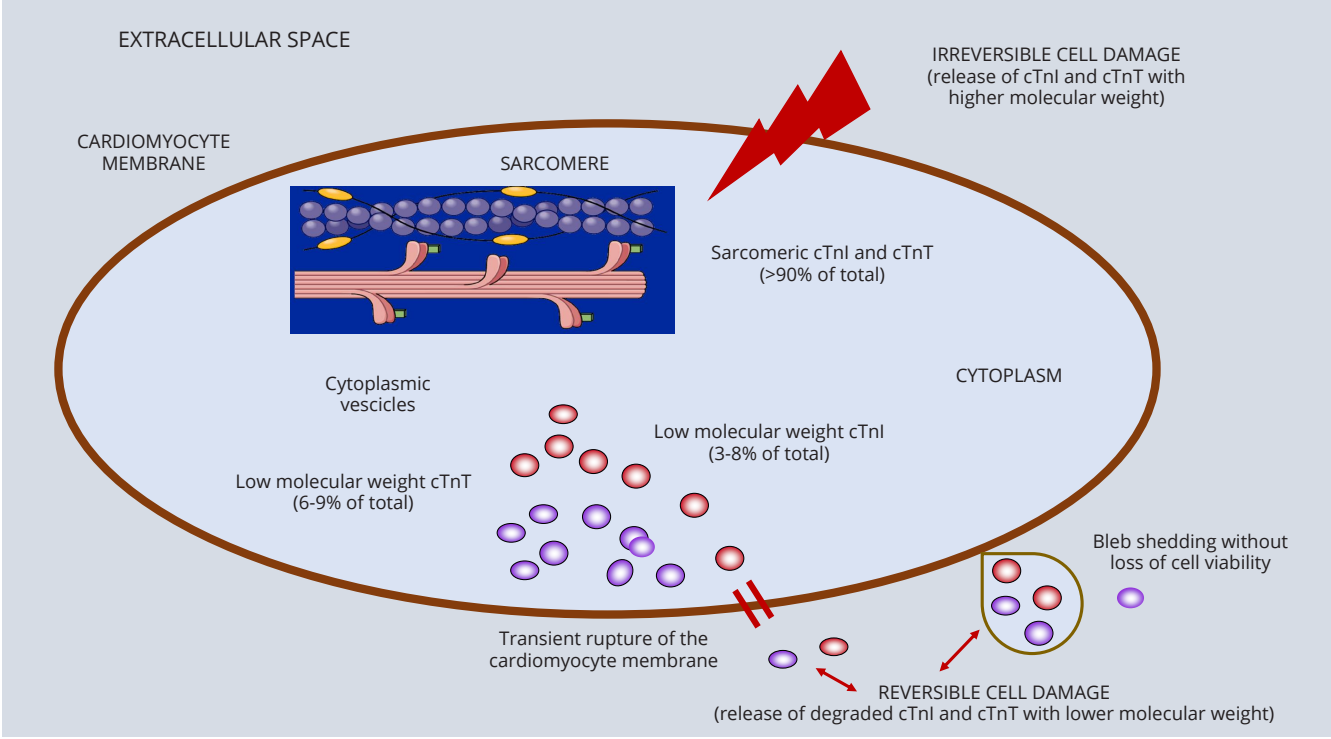

Figure 2.—The figure illustrates the current concepts of potential cardiac troponin release mechanisms from injured myocardium.

roptosis.[58, 61, 107-115] Currently, it has been demonstrated that under the same cardiovascular disease model, there are different programmed cell death pathways as well as complex molecular-level interconnections.[107-113]

Actually, the link between programmed cell death of the cardiomyocytes in patients with cardiovascular disease is not fully understood.[109-113] However, some authors have recently suggested that a programmed cell death mechanism named PANoptosis represents an innovative, integrated view playing an important role even in the field of the cardiovascular diseases.[112, 113] PANoptosis is a newly defined programmed cell death involving some specific and different death mechanisms, including apoptosis, pyroptosis and necroptosis.[112, 113] Recent studies have indicated that the pathophysiological mechanisms related to PANoptosis may play a pivotal role in modulating immune responses and inflammatory processes, and in determining cell fate involved in the pathogenesis of several acute or chronic diseases.[109-115] At the molecular-level, the PANoptosis mechanism may interconnect the different programmed cell death pathways under the same cardiovascular disease model.[112, 113] More specifically, the PANoptosis pathway may involve apoptotic proteins, inflammasomes and necroptotic elements, suggesting a complex regulatory network that could play a pivotal role in the evolution of some common cardiovascular pathologies, such as atherosclerotic plaque formation or response to ischemia-reperfusion injury and infarction in myocardial tissues.[109-115]

Take-home messages

- Several pathophysiological mechanisms can produce either reversible or irreversible damage on cardiomyocytes, depending from the intensity and the duration of injury;[1, 36-40, 43]
- a reversible damage is promptly repaired by the cardiomyocyte, allowing it to quickly resume normal function. In contrast, irreversible damage leads to cardiomyocyte death;[36-40, 43]
- cardiomyocyte death can occur *via* various mechanisms, both programmed and non-programmed (such as necrosis);[36, 39, 40, 55-58, 61, 107-112]
- current evidence suggests that multiple mecha-

nisms contribute to programmed cell death of cardiomyocytes in patients with cardiovascular diseases, including apoptosis, autophagy, necroptosis, pyroptosis, and ferroptosis.[58, 61, 107-115]

From myocardial injury (MI) to acute myocardial infarction (AMI)

Since the year 2000, the consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction stated that cTnI and cTnT were to be considered the biomarkers of choice for the diagnosis of AMI (217). In particular, these guidelines recommended that cTnI and cTnT, measured with immunoassay methods, should be considered the biomarkers of choice for the differential diagnosis of Acute Coronary Syndrome (ACS), and that the 99th URL value for cardiac troponins should be measured with an imprecision of ≤10 CV%.[116]

In August 2018, the Fourth Universal Definition of Myocardial Infarction[1] stated that: "*the detection of an elevated cTn value above the 99th percentile URL value is defined as myocardial injury (MI).*" Moreover, the same document specified that the MI is considered acute if there is a rise and/or fall in circulating levels of cTnI or cTnT.[1] In addition, this document stated that the clinical diagnosis of AMI requires the preliminary detection of an acute MI by means of cTn methods (preferably hs-cTnI and hs-cTnT assays) in the setting of the clinical evidence of the acute myocardial ischemia.[1] In particular, the temporal biomarker variations should be taken into considerations only if the clinical setting of acute myocardial ischemia is confirmed by some clinical criteria, including:

- clinical symptoms of myocardial ischemia;
- new ischemic ECG changes;
- development of pathological Q-waves;
- imaging evidence of a new loss of viable myocardium or a new regional wall motion abnormality in a pattern consistent with an ischemic etiology;
- identification of a coronary thrombus by angiography or autopsy.

Furthermore, the evidence of an imbalance between myocardial oxygen supply and demand unrelated to acute athero-thrombosis, meets criteria for type 2 AMI.[1] These statements have been confirmed by all the most recent national and international guidelines.[2-4, 7, 117-123]

It is well known that the MI can occur in different cardiac and non-cardiac diseases.[1] Accordingly, several potential confounding factors must be considered when evaluating a patient presenting with chest pain to the ED.[1, 2, 117-123] In accordance with the Fourth Universal Definition of Myocardial Infarction, a temporal variation of the biomarker levels is required to demonstrate the presence of an acute MI.[1] Therefore, all the guidelines actually recommend standardized clinical algorithms based on temporal changes in circulating hs-cTn levels to detect myocardial injury progressing to AMI.[1, 2, 117-123]

More specifically, in August 2020, the European Society of Cardiology (ESC) guidelines strongly suggested that clinical algorithms based on temporal changes of less than 3 hours, using hs-cTn methods, should be preferred for the diagnosis of ACS in patients presenting without persistent ST-segment elevation (NSTE-ACS).[117] These recommendations are based on the clinical observation that a more rapid evaluation of patients with a low probability of AMI may allow a more efficient and rapid management of the patients admitted to the ED.[117, 120-126] Furthermore, several recent studies have demonstrated that a more rapid AMI diagnosis allows to significantly reduce healthcare costs of AMI patients by enabling an early and specific treatment improving outcomes.[117, 120-126]

Although significant progress has been made in recent years, some important questions remain regarding the pathophysiological interpretations of hs-cTnI and hs-cTnT results in routine clinical practice.[3, 6-9, 17, 43, 117-123] Several recent studies have shown that some forms of cTnI and cTnT with reduced MW and faster plasma clearance than sarcomere-bound troponins, are often measured after intense and prolonged physical exercise in apparently healthy subjects and even in well-trained athletes (*e.g.*, after a marathon or cycling race).[6, 7, 9, 41-43, 52, 53] In addition, higher levels of degraded forms of cTnI and cTnT (*i.e.*, over the 99th percentile URL value) are frequently measured in patients with acute or chronic renal diseases.[49] Because the current commercially available hs-cTnI and hs-cTnT methods are not able to directly measure and identify the biomarker molecular forms with lower MW, increased hs-cTnI and hs-cTnT values over the 99th percentile URL value can be frequently measured in apparently healthy subjects after intense physical activity or in patients with reduced renal function.[6, 7, 9, 41-43, 52, 53] In conclusion, a single biomarker measurement using the currently available hs-cTnI and hs-cTnT methods cannot demonstrate whether the increased biomarker levels are due to a myocardial injury, and/or to a reduced renal clearance of biomarker in some pathophysiological clinical

conditions, or even to a presence of an analytical interference in immunoassay methods.[6, 7, 9, 17, 23, 41-43, 52, 53, 127, 128]

Take-home messages

- The Fourth Universal Definition of Myocardial Infarction[1] emphasizes that the clinical diagnosis of AMI requires the preliminary detection of an acute MI by means of cardiac troponin assay (preferably hs-cTnI and hs-cTnT assays) in the setting of clinical evidence of acute myocardial ischemia;
- in August 2020, the European Society of Cardiology (ESC) guidelines recommended that diagnostic algorithms using hs-cTn assays with sampling intervals of less than 3 hours (better 1-2 hours) be preferred for diagnosing ACS in patients presenting with NSTE-ACS;[117]
- these recommendations stem from clinical findings showing that rapid patient evaluation, particularly in those with a low probability of AMI, enables more efficient and expedited management of patients in the ED;[117, 120-125]
- a single measurement of hs-cTnI or hs-cTnT cannot reliably distinguish whether elevated biomarker levels are attributable to myocardial injury, reduced renal clearance, or analytical interferences in the immunoassay methods used, especially under certain pathophysiological conditions.[6, 7, 9, 17, 23, 41-43, 52, 53, 127, 128]

Clinical considerations

Role of cardiac biomarker variations in clinical practice

Several studies have recently demonstrated that the variation of hs-cTnI and hs-cTnT levels is essential not only for the diagnosis of AMI in patients admitted to ED for thoracic pain, but also for the evaluation of the cardiovascular risk in some frequent clinical conditions, such as: general population,[6, 7, 69, 93, 96-107] athletes after intense physical activity,[6, 7, 41, 43, 52] patients submitted to cardiac and non-cardiac major surgery,[129-147] pregnant women, especially in presence of some complications (*i.e.*, diabetes mellitus, hypertension, eclampsia or cardiomyopathy).[148-155]

In these clinical conditions, it is important to accurately evaluate the individual biological variation, defined as the random fluctuations of a biomarker (*i,e.*, hs-cTnI and hs-cTnT circulating levels) around the individual homeostatic set point.[6, 69, 72] This parameter is generally expressed as CVi calculated using at least two measured hs-cTnI and hs-cTnT values, assuming that this index is relatively constant in apparently healthy individuals or in patients with stable disease.[6, 69, 72]

TABLE II.—*RCV values for hs-cTnI and hs-cTnT methods in the range from 5 ng/L to 40 ng/L.*

Methods	RCV % (mean±SE)	RCV % (min-max)
ARCHITECT hs-cTnI	31.3 (1.5)	28.4-36.9
ACCESS hs-cTnI	31.0 (1.9)	27.5-38.1
ADVIA XPT hs-cTnI	33.4 (3.0)	27.4-43.8
ECLIA hs-cTnT	32.4 (3.5)	26.0-45.4
Global mean (SE)	32.0 (0.5)	26.0-45.4

RCV% values were calculated in a reference laboratory using standardized protocols, as detailed previously.[29, 32, 33, 69, 156, 157]
SE: Standard error.

Taking into account these evidences, some recent documents, prepared by the Italian Study Group on Cardiac Biomarkers,[6, 7, 9, 69, 121] have suggested a simple thumb rule to evaluate the temporal variations of hs-cTnI and cTnT circulating levels. For the diagnosis of NSTE-ACS, these documents[6, 7, 9, 69, 121] suggest to take into consideration the Reference Change Value (RCV) of hs-cTnI and hs-cTnT methods.[72] The RCV, also known as a critical difference, is a calculated value used to determine if a change between two sequential test results for a patient is significant, meaning it's unlikely to be due to random variation.[72]

The RCV is usually calculated using the following classical mathematical formula, according to Fraser GC:[72]

$$RCV = 2^{1/2} \times Z \times [(CVa)^2 + (CVi)^2]^{1/2}$$

where: CVa is the value of analytical variability of the hs-cTnI or hs-cTnT method (*i.e.*, the imprecision expressed as a coefficient of variation, CV%) (Figure 1, Table I); CVi is the intra-individual variability of the subject/patient (expressed as CV%); Z is the value of Zeta Score for a bidirectional probability of 95% (usually equal to 1.96).[72]

As an example, the mean RCV values related to some popular hs-cTnI and hs-cTnT methods are summarized in Table II, as previously reported in detail by several studies.[29, 32, 33, 69, 156, 157] These data indicate that the RCV values can vary on average by 32% (with a minimum of approximately 25% and a maximum of approximately 45%), considering the range of hs-cTnI and hs-cTnT from 5 ng/L to 40 ng/L. More specifically, these data suggest that a percent increase (or decrease) >30% between two concentrations (expressed as ng/L) measured with the same hs-cTnI or hs-cTnT methods, calculated using two consecutive samples collected from the same patient,

should be considered to be significant for the presence of AMI.[6, 7, 9, 69, 121] This simple thumb rule directly derives from the data reported in Table I and Figure 1. These data demonstrate that: all hs-cTnI and hs-cTnT methods show cut-off values for the diagnosis of MI measured with a CV<10%,[6, 9, 23-25, 27-34] as recommended by all the national and international guidelines[1-4, 7, 20] and that the imprecision profile of hs-cTnI and hs-cTnT methods closely interpolate similar reciprocal functions.

Conversely, the ESC 2020 guidelines[117] recommend the use of rapid "rule-in" and "rule-out" algorithms, based on temporal variations of hs-cTnI and hs-cTnT concentrations for the diagnosis of NSTE-ACS in patients admitted to ED with acute thoracic pain. More specifically, the ESC 2020 guidelines suggest to calculate the difference in hs-cTnI/hs-cTnT concentrations (defined as delta changes and expressed as ng/L), measured in two samples collected from patients admitted to ED for the diagnosis of NSTE-ACS, by taking into account to two different temporal intervals: the 0 h/1 h algorithm, suggested as the best option, including two blood samples collected at admission to ED (0 time) and after 1 h or the 0 h/2 h algorithm, suggested as the second option, including two blood samples collected at admission and after 2 hours.[117] It is important to note that the delta changes values suggested by ESC 2020 guidelines[117] are strictly based on analytical characteristics and performances of cardiac troponins immunoassays, in particular the LoD and the 99th percentile URL values of the hs-cTnI and hs-cTnT methods.[9, 118, 119, 121]

It is well known that the analytical characteristics of hs-cTnI and hs-cTnT immunoassays are specific and greatly different among methods.[2-4, 6, 9, 20, 23-26] Therefore, the clinical interpretation and memorization of data concerning the delta change values, suggested for temporal algorithms by 2020 ESC guidelines for the diagnosis of NSTE-ACS,[117] is challenging because the suggested delta change values differ significantly according to:

- the analytical performances of different immunoassay methods;
- the temporal algorithms suggested by guidelines (*i.e.*, 0-1 h hour *vs.* 0-2 hours);
- the respective levels of hs-cTnI or hs-cTnT (ng/L) measured in the patient sample;
- the clinical characteristics of the patients, including: age, sex, ethnicity, clinical history and presence of comorbidity;
- the timing of presentation to ED of the patients (for example: early *vs.* late presentation).[6, 7, 41, 43, 52, 118-124]

Finally, the algorithms suggested by the 2020 ESC guidelines for the diagnosis of NSTE-ACS[117] have not been recently updated. Accordingly, the data related to the most recent versions of the previously or new commercially available hs-cTnI and hs-cTnT methods (in particular including some POCT methods) are not taken into consideration in the algorithms proposed by ESC 2020 guidelines.[6, 9]

On the contrary, the evaluation of the temporal variations of hs-cTnI and hs-cTnT circulating levels, as a percent increase (or decrease) in the biomarker concentration ≥30%, allows us to evaluate as significant the variation observed between two biomarker measurements in two consecutive sample of the same patient.[6, 7, 9, 69, 121] This 30% rule can be used to evaluate the clinical significance of the biomarker variations using all the hs-cTnI or hs-cTnT methods currently available, not only in patients with thoracic pain admitted to ED, but also in several pathophysiological conditions (*i.e.*, evaluation of cardiovascular risk in general population, pregnant women, athletes after strenuous physical exercise, and patients undergoing non-cardiac surgery).[6, 7, 9, 69, 147, 155] Finally, it must be underlined that the 30% variation between two consecutive results in the same patient, may be adopted for comparing the values obtained with the same hs-cTnI or hs-cTnT method, possibly in the same clinical laboratory or using the same POCT method at the patient's bedside.

Take-home messages

- Owing to the low individual index of biological variability of cardiac troponins (average ≤0.3), hs-cTnI and hs-cTnT assays are particularly well-suited for serial monitoring of biomarker changes in individual patients;[6, 7, 9, 69-71]
- some recent documents from the Italian Study Group on Cardiac Biomarkers have proposed a simple rule for interpreting temporal variations in hs-cTnI and hs-cTnT circulating levels;[6, 7, 9, 69, 121]
- specifically, these guidelines recommend that a ≥30% increase or decrease in biomarker concentration should be considered clinically significant for diagnosing AMI, in accordance with the Fourth Universal Definition of Myocardial Infarction;[1]
- this 30% change should be calculated based on two consecutive hs-cTnI or hs-cTnT measurements collected from the same patient;[6, 7, 9, 69, 121]
- the 30% rule is applicable not only for patients

presenting with chest pain in the ED but also across a variety of pathophysiological conditions, such as: cardiovascular risk assessment in the general population, pregnant women, athletes after strenuous physical exercise, and patients undergoing non-cardiac surgery.[6, 7, 9, 70, 149, 157]

Evaluation of hs-cTnI and hs-cTnT in patients with renal diseases

Several Authors have remarked the fundamental role played by renal function on circulating cTnI and cTnT levels in both healthy subjects or patients with cardiovascular diseases and/or other comorbidities, especially acute and CKD.[17, 44, 49-51, 69, 158-185] Some specific analytical, pathophysiological and clinical issues are needed in order to better understand the mechanisms related to variations of cTnI and cTnT levels observed in patients with reduced renal function.[6, 7, 9, 69, 70, 158-185] First, cTnI and cTnT methods should be evaluated for the matrix effect due to the use of urine sample; furthermore, considering the great difference in specificity and sensitivity among the performance of immunoassay methods, only the hs-cTnI and hs-cTnT methods with analytical performances validated using standardized protocols, as reported in reports, documents and recommendations from a number of international national and international societies of laboratory medicine.[2-4, 9, 20, 26, 127] Finally, the results found in patients with cardiovascular diseases, but with conserved renal function, should be compared with those with both compromised cardiac and renal functions.

hs-cTnI and hs-cTnT assay in urine samples

Some studies aimed to evaluate the pathophysiological and diagnostic role of cTnI and cTnT measurement in urine samples in healthy subjects or patients with cardiovascular and/or kidney diseases with different severity in renal function impairment are available.[175-180] In 2018, Streng *et al.*[175] provided evidence that cTnT molecules can be identified with data-dependent tandem mass spectrometry assay (t-SIM/dd-MS2) in urine samples from 20 patients with AMI. The urine samples were treated using a cTnT-specific immunoprecipitation technique and a non-specific acetonitrile protein precipitation. After in-solution digestion of the precipitated proteins, the resulting peptides were separated and analyzed using HPLC and mass spectrometry with a targeted selected ion monitoring assay with t-SIM/dd-MS2.[175] The t-SIM/dd-MS2 assay was validated using a synthetic peptide standard containing 10 specific cTnT peptides of interest and with purified human intact cTnT spiked in urine from healthy individuals. Using this assay, six different cTnT-specific peptides were identified in urine samples from three different AMI patients.[175] As observed by Authors, the results of this study demonstrated that some degraded cTnT peptides are present in urine samples in AMI patients showing high biomarker concentrations in serum (>1000 ng/L) and concomitant proteinuria.[175]

Other recent studies reported that both cTnI and cTnT can be detected by immunometric methods in human urine samples.[176-180] However, an accurate cTnI or cTnT measurement in urine samples without a preliminary extraction followed by chromatographic purification and/or concentration is very difficult because of the very low concentrations of cardiac troponins in urine samples (especially in healthy subjects or patients without cardiac diseases), the presence of several degraded and/or complexed forms of cTnI and cTnT molecules, and the possible assay interference in the immunometric methods due to the acidic nature of pH in urine samples.[10-12, 15, 17, 176-180] These analytical drawbacks explain why accurate data on the renal clearance values of cTnI and cTnT in healthy adult subjects are not available so far. Indeed, actually, only few results obtained in urine samples from children or adult patients with cardiovascular diseases, containing high urinary hs-cTnI and hs-cTnT concentrations have been published.[176-180]

It is well known that some cardiomyopathies and congenital heart defects represent the primary cause of increased of hs-cTnI and hs-cTnT in children.[76, 80] The results of some recent studies confirmed that the measurement of circulating hs-cTnI and hs-cTnT levels is useful for the diagnosis and management of cardiovascular disorders in children.[76, 80] In particular, two recently published papers from the group of Bakoš *et al.*[176, 177] have reported some interesting results related the hs-cTnI and hs-cTnT levels in urine samples of children with congenital heart defects. The principal aims of these studies are to better clarify the elimination process of cardiac troponins from the bloodstream and to evaluate the possible clinical role of cardiac biomarkers in urine samples in children with congenital heart diseases.

In particular, the first study by Bakoš *et al.*[176] aimed to investigate the variation of serum and urine hs-cTnT levels and its correlation in 90 infants or children below 24 months of age (58 male, 32 female), including both

healthy subjects and some patients with congenital heart defects. The experimental group consisted of 48 children with ventricular septal defect (VSD) who underwent cardiac surgery (group 1). Two control groups were involved in this study: the first control group consisted of 18 infants after extracardiac formation of bidirectional cavopulmonary connection (BCPC) surgery (group 2), while the second control group consisted of 23 healthy infants, in whom the absence of congenital heart defects was confirmed by echocardiography (group 3). In these children, the hs-cTnT values (Roche Troponin T (hs) STAT, REF 05092728 190, Roche Diagnostics, Mannheim, Germany) have been measured in serum and urine at five time points: the first sample was taken on the day before cardiac surgery, while the other four samples were measured immediately after surgery, and on the first, third, and fifth postoperative days. Only one urine samples (*i.e.*, the first morning urine sample) was collected for determining hs-cTnT in the control group of healthy infants. A statistically significant negative correlation was found between serum and urine hs-TnT concentrations and GFR calculated by creatinine clearance. Patients who underwent surgical repair of VSD had significantly higher concentrations of hs-cTnT in serum on the first three postoperative measurements compared to those who had BCPC surgery.[176] Urine hs-TnT values measured pre-operatively in children undergoing BCPC surgery was higher (median 7.3 ng/L, IQR 6.6-13.3) compared to those of children undergoing VSD surgery (median 6.5 ng/L, IQR 4.4-8.9) as well as to those in healthy population (median 5.5 ng/L, IQR 5.1-6.7).[176] However, after logarithmic transformation, there was no statistically significant difference (according to ANOVA) in urine hs-TnT concentrations between groups at any point of measurement in pre- or post-operative samples. Overall, the results of this study suggest that urine samples appear to be a less valid sample in comparison to the serum for the assessment of post-operative myocardial damage in children using the hs-cTnT method.[176]

The specific aim of the second study by Bakoš *et al.*[177] was to explore the role of hs-cTnI as indicator of cardiac damage in sick children with some congenital heart diseases. Authors enrolled a total of 70 children under 24 months of age, divided in 3 different in groups including healthy or sick children: group 1, 38 children who underwent cardiac surgery for VSD repair; group 2, nine infants before and after the surgical formation of BCPC; group 3, 23 healthy children without cardiovascular or other acute or chronic diseases (considered as a control group).[177] Both urine and serum samples were assessed on four occasions with hs-cTnI method in the two groups of children with congenital heart diseases (*i.e.*, VSD and BCPC groups). Conversely, the third group, underwent only a single hs-cTnI measurement in urine samples, because no blood samples were taken for ethical reasons.[177] As expected from previously reported results,[75, 76, 80] the children who underwent cardiac surgery showed higher serum hs-cTnI values in the early postoperative period, followed by a progressive reduction to lower levels in the post- operative days.[177] Moreover, significantly higher serum hs-cTnI values were observed in the first group of children compared to second one ($p<0.05$). On the contrary, authors reported no significant increase in urine hs-cTnI values directly related to myocardial damage. In fact, the hs-cTnI levels were not measurable in most urine samples, even if the method adopted in this study (CMIA ARCHITECT STAT high-sensitive Troponin-I, Abbott Diagnostics) is a high-sensitivity method.[26, 177] In this regard, the authors suggest that potential explanatory factors for the failure to detect the biomarker levels in urine samples may include the isoelectric point of cTnI, elevated urinary concentrations of salts and urea, variations in urine acidity (different pH levels), and a relatively low cTnI concentration in urine.[177]

Overall, the results of these two studies[176, 177] indicate that at present time the assay of biomarker concentrations in urine samples using commercially methods for hs-cTnI and hs-cTnT assay should not be recommended for an accurate assessment of cardiac damage in infant and children with congenital cardiovascular diseases. However, it is important to note that the hs-cTnI and hs-cTnT methods used in the studies by Bakoš *et al.*[176, 177] are not validated by the manufacturers for the measurement in urine samples. Moreover, these results[176, 177] strongly suggest that further studies are needed in order to improve the analytical performance of hs-cTnI and hs-cTnT methods to accurately evaluate the biomarker levels in urine samples collected from infant and children with congenital cardiovascular diseases.[75, 76, 80]

Some studies have taken into considerations the pathophysiological and clinical role of hs-cTnI measurement in urine samples of healthy adults and patients with cardiovascular diseases and/or comorbidities.[178-180] In 2018, Pervan *et al.*[178] aimed to establish preliminary reference intervals for hs-cTnI concentrations in urine of healthy adults in Croatia. 60 healthy subjects (M/F ratio = 1, age range from 24 to 63 years) are selected using the following criteria: non-smoker, aged 25-65 years, BMI <30 kg/m^2, absence of acute and chronic diseases (*i.e.*, heart disease and thyroid disease, hypertension, hyperlipidemia), no severe

physical activity for the last 7 days, no night work during the last 30 days.[178] The cTnI concentrations in random urine samples were determined by the hs-cTnI ARCHITECT Abbott method using a urine volume of 160 mL, as suggested by the manufacturer for blood samples.[26, 178] The measured hs-cTnI values in urine samples were above the detection limit (LoD 1.2 ng/L) in 59 (98.3%) subjects and above the quantification limit (LoQ 4.7 ng/L) in 51 (85.0%) subjects.[178] The preliminary 99th percentile URL values in random urine samples of the selected reference persons from Croatia were 39.3 ng/L for males and 35.2 ng/L for females, respectively. Authors concluded that the obtained results indicated that the cTnI is removed from the blood *via* kidneys and can be determined in the urine by some hs-cTnI methods currently used to determine the biomarker in plasma.[178]

In 2020, Chen *et al.*[179] investigated the accuracy of urine hs-TnI measurement as predictor for incident cardiovascular events in patients with diabetes mellitus (DM). Authors reported that the hs-TnI levels were measured in fresh urine samples using a mini-VIDAS analyzer (Bio-Mérieux SA, Mercy I'Etoile, France) showing a CV ≤10% with detectable range from 0.75 to 40,000 ng/L.[179] For values exceeding this range, the urine samples were diluted with normal saline. The study cohort included 378 patients (age 68.1 [11.0] years; M/F:65.6%), with diagnosis of type 2 DM, enrolled and treated in a cardiovascular outpatient hospital department from December 2018 to December 2019.[179] In particular, the enrolled patients showed several co-morbidities, including: artery hypertension 72.8%, coronary artery disease 43.9%, heart failure (ejection fraction <50%) 17.5%, atrial fibrillation 24.9%, and CKD 9.6%. The authors observed significantly higher hs-cTnI levels in urine samples collected from patients with incident cardiovascular events.[179] The multivariate logistic regression analysis using different models consistently showed that a value of hs-TnI >4.1 ng/L measured in urine samples was an independent predictive factor of incident cardiovascular events. Moreover, the optimal cutoff value for urinary hs-TnI to predict incident cardiovascular events (p=0.027) was 1.55 ng/L, corresponding to an area ROC-AUC of 0.611. The Authors concluded that a single measurement of urinary hs-TnI may be an acceptable biomarker for predicting subsequent incident cardiovascular events in patients with DM.[179]

More recently, Svagusa *et al.*[180] aimed to compare the concentration of cTnI in the first morning urine samples collected from both patients with severe aortic stenosis and healthy population. The main aim of collecting samples from the healthy population was to calculate the reference values for the first morning urinary troponin. The blood and first morning urine samples have been collected from 34 healthy control individuals (17 female; median age 34.0, IQR 28.0–44.0; F/M ratio 50.0) and 25 patients with severe aortic stenosis (15 female; median age 70.5, IQR 64-75; F/M ratio 55.6) before surgical treatment.[180] The cTnI and cTnT concentrations were determined using the hs-cTnI (CMIA, ARCHITECT High Sensitive Troponin-I, Abbott Diagnostics) and hs-cTnT (hs-Troponin T, Roche Diagnostics) methods.[26] It is important to note that biomarker concentrations were measured with the same analytical protocols suggested by manufacturers for the hs-cTnI or hs-cTnT measurement in serum or plasma samples. Patients with severe aortic stenosis had significantly lower hs-cTnI values (p<0.001) in the first morning urine samples (median 0.3 ng/L, IQR 0.1-0.6) in comparison to those of healthy population (mean 15.2 ng/L, IQR 8.4-19.9).[180] On the other and, both hs-cTnI and hsTnT plasma concentrations were significantly higher [p<0.001] in patients with severe aortic stenosis compare to controls [hs-cTnI, patients: median 8.1 ng/L, IQR 5.4-17.5; controls: median 0.9 ng/L, IQR 0.5-1.3; hs-cTnT, patients: median 15.2 ng/L, IQR 9.9-23.5; controls median 3.4 ng/L, IQR 3.0-5.5).[180] In particular, in patients with severe aortic stenosis, plasma hs-cTnI values were, on average, 27 times higher than those measured in the first morning urine samples; on the contrary, hs-cTnT values in urine samples were, on average, 1.8 times higher than those measured in plasma samples.[180] On the other hand, considering the healthy subjects, the hs-cTnI values in the first morning urine were, on average, 16.9 times higher than in the plasma, while the hs-cTnT values in healthy participants were, on average, 2.6 times higher in the first morning urine than in the plasma, being this a result comparable to that observed in patients with severe aortic stenosis.[180]

These results[176-180] concerning the hs-cTnI and hs-cTnT values measured in both blood and urine samples, suggest some analytical, pathophysiological and clinical considerations. From an analytical point of view, all these studies show significant limitation concerning the analytical protocols used for the assay in urine samples. In fact, the hs-cTnI and hs-cTnT methods used in these studies, are not (specifically) validated for the measurement of the biomarkers in urine samples.[176-180] Accordingly, some preliminary validation studies are needed in order to set up the analytical protocols allowing an accurate measurement of the biomarker levels in urine samples, from both healthy subjects and patients with cardiovascular diseases and/or

other comorbidities. In particular, these analytical studies should verify some specific analytical aspects for the measurement of hs-cTnI and hs-cTnT in urine samples in order to overcome some criticisms related to: the possible influence of lower pH of urine on immunoassay methods, the interferences related to some degraded forms of the biomarkers in urine samples and the calculation of the recovery of cTnI and cTnT standard preparations in urine samples.[22, 23]

Another relevant criticism is that all the clinical studies evaluating the measurement of hs-cTnI and hs-cTnT in urine samples from healthy subjects or patients with cardiovascular and/or other co-morbidities, have the strong limitation related the low number of healthy subjects or patients studied.[176-180] Accordingly, all the most recent review articles, expert's documents and guidelines suggest that additional studies are needed to clarify the pathophysiological mechanisms involved the renal clearance of cardiac biomarkers.[6, 8, 9, 21, 128, 170] Therefore, it is urgently needed to set up specific clinical studies in order to assess both reference and clinical cut-off values for all the hs-cTnI and hs-cTnT methods in urine samples collected from large cohort of the reference populations.

Diagnostic role of hs-cTnI and hs-cTnT measurement in patients with impaired renal function

Several studies have evaluated the measurement of circulating levels hs-cTnI and hs-cTnT levels in CKD patients.[15]

In 2015, Chung *et al.*[15] investigated the relationship between renal function and circulating hs-cTnT levels in a large population of patients (17,113 individuals) with available data including both hs-cTnT and creatinine results. The clinical data of this population were extracted from the laboratory database representing a continuous 17-month period. The hs-cTnT and creatinine results were available from 10,418 patients, with 49% of the requests from the ED. After applying exclusion criteria, 3108 sequential pairs result were available from patients who had undergone repeated testing, in a median time between results of 15 h, IQR 7-25. A moderate reduction in GFR (30-59 mL/min/1.73 m^2) corresponded to a median hs-cTnT value above the cut-off of 14 ng/L (*i.e*. the 99th percentile URL value). Considering the modest association found between changes in hs-cTnT and creatinine within individuals in the short term, authors suggested the need for caution when interpreting hs-cTnT elevations in patients with end stage chronic kidney disease.[15] However, considering the relevant diagnostic role of the biomarker testing, the interpretation of the variations in circulating hs-cTnI and hs-cTnT levels in CKD patients with kidney impairment is particularly challenging, because it is theoretically conceivable that the circulating biomarker levels may be increased because of: the presence of a myocardial injury, a reduced renal clearance, or both conditions.[174, 182-186]

In 2018, Kavsak *et al.*[174] reported that both hs-cTnI and hs-cTnI levels were negatively correlated with GFR in 1212 patients admitted to ED with symptoms suggestive of ACS. In particular, in the lowest eGFR category (<30 mL/min/1.73 m^2) no patient had a concentration below the LoD of the assays (*i.e*., the lowest hs-cTnT was 7 ng/L [LoD <5 ng/L] and hs-cTnI was 3 ng/L [LoD <2 ng/L]).[174] The Authors concluded that the highest combined sensitivity (100%), Negative Predicted Value (100%) and proportion of low-risk for AMI (45% of group) was observed for patients with hs-cTnT <6 ng/L with an eGFR ≥90. The clinical performance of hs-cTnI and hs-cTnT in diagnosing or ruling-out an acute cardiac event (*i.e*., the presence/absence of a myocardial injury) can vary according to eGFR values; accordingly, accurate cardiovascular risk stratification in patients with impair renal function should requires knowledge of the eGFR.[174]

More recently, Gallacher *et al.*[184] described the clinical usefulness of hs-cTnI testing on the diagnosis, management, and outcomes of myocardial infarction in patients with and without kidney impairment. The diagnosis and primary outcome of AMI or cardiovascular death after one year were compared in patients with and without kidney impairment. The study enrolled consecutive 49,927 patients (mean age 61 years; 47% women) attending the ED with available creatine concentrations and suspected to having ACS.[184] All these patients underwent cardiac troponin testing at presentation and 6 to 12 hours after the onset of symptoms at the discretion of the attending clinician according to international guidelines.[1, 184] All patients underwent testing with both contemporary cTnI method (ARCHITECT STAT troponin I, Abbott Laboratories) and hs-cTnI (ARCHITECT STAT high-sensitive troponin I, Abbott Laboratories) methods.[184] Cardiac troponin concentrations were elevated in 46% (4220 of 9080) and 16% (5891 of 37,847) of patients with and without kidney impairment, respectively (p<0.001).[184] Of the 10,111 patients (71 [SD: 15] years, 48% women) with elevated cardiac troponin concentrations, 42% (4220 of 10,111) had kidney impairment. The use of the hs-cTnI method reclassified 17% (1717 of 10,111) of patients with myocardial injury or AMI who were not identified by the contemporary cTnI assay.[184] Kidney impairment was present in 41% (3428 of 8394) of the patients identified by the contemporary as-

say and in 46% (792 of 1717) of patients reclassified by the hs-cTnI method (p<0.001). These patients were older, more likely to be women, and less likely present chest pain than patients with normal kidney function. In patients with hs-cTnI above the 99th percentile URL value, the primary outcome occurred twice as in those with kidney impairment in comparison to those without (24% *vs.* 12%, hazard ratio 1.53, 95% CI 1.31 to 1.78).[184] The Authors concluded that increased hs-cTnI concentrations were threefold more frequent in patients with kidney impairment than in those with normal kidney function. The use of hs-cTnI method increased the diagnosis of type 1 AMI in patients with and without kidney impairment.[184]

It is well known that cardiovascular disease is a major complication of CKD and a leading cause of death in patients, with a high prevalence of recurrent hospitalizations, debilitating symptoms, and lower health-related quality of life.[185-188] Moreover, data from multiple epidemiologic and cohort studies as well as intervention studies suggest that CKD is an independent risk factor for cardiovascular disease and that patients with advanced CKD stages (CKD stages 4-5) are at significantly higher risk of cardiovascular disease; actually, sudden cardiac death is the largest single cause of death in chronic dialysis patients.[185-188] The USA Renal Data System (USRDS) 2018 Annual Data Report reported that the prevalence of AMI in the hemodialysis population is 15.3%.[189, 190] Considering the patients undergoing hemodialysis for CKD, several epidemiological and clinical studies data has shown that AMI accounts for the main reason for death in these patients.[186-188] The diagnosis of AMI in patients with CKD is difficult because these patients usually have increased hs-cTnI and hs-cTnT concentrations.[181-188] Moreover, it is also well known that patients on long-term dialysis show poor survival after AMI.[186-192]

In 2018, Kraus *et al.*[193] aimed to describe a diagnostic algorithm for suspected NSTE-AMI using hs-cTnI and hs-cTnT measurements in patients with acute chest pain, divided into two groups according to the presence/absence of CKD. The patients/cases have been enrolled from two different cohorts, including: 1494 patients from a prospective cohort study in which hs-cTnI measurements are available and 7059 cases from a clinical registry with hs-cTnT measurements. The first prospective cohort included 280 CKD patients (mean eGFR 46 [SD: 12] mL/min/1.73 m^2), while the second registry data set contained 1581 CKD patients (mean eGFR 19.4 mL/min/1.73 m^2).[193] In both cohorts, CKD patients were more likely to have adjudicated NSTE-AMI than non-CKD patients. Overall, the clinical performance to detect NSTE-AMI using hs-cTnI and hs-cTnT assays was reduced in CKD *vs.* non-CKD patients (ROC and AUC analysis: 0.82 *vs.* 0.91 for hs-cTnI and 0.26 *vs.* 0.73 for hs-cTnT respectively).[193] However, authors suggest that the clinical performance can be significantly improved by applying optimized cutoffs to either the first or the second measurement after 3 hours. The best diagnostic performance to rule-in or rule-out AMI was achieved with an algorithm including serial biomarker measurements in 69% (using the hs-cTnI method) and 55% (using the hs-cTnT method) of CKD patients, respectively.[191] Interestingly, the results of this study confirm that the evaluation of serial changes in hs-cTnI/hs-cTnT concentrations can improve diagnostic accuracy for AMI even in CKD patients, due to the very low intra-individual biological variation of the biomarker circulating levels,[191] as previously observed in patients with cardiovascular or non-cardiovascular diseases.[6, 69-71, 121, 147, 155]

More recently, Knott *et al.*[194] aimed to re-evaluate the diagnostic performance of hs-cTnT assay in patients with and without CKD, admitted to ED for AMI diagnosis, using the diagnostic algorithms for ruling-in AMI, according to the recommendations suggested by ESC 2020 guidelines.[117] Furthermore, the short- and long-term outcomes of AMI patients with and without CKD were also examined.[192] The study cohort included 1992 patients (women 52%; mean age 62 [SD: 18] years); including 501 CKD patients (25% of total; women 53%; mean age 76 [SD: 13] years). In this study, the AMI diagnosis (type 1 or 2) was adjudicated in 155 patients (8% of total number of enrolled patients), including 80 without CKD (5% of patients), and 75 with CKD (15% of patients, p<0.001.[194] The myocardial injury was detected in 701 patients (35% of the total number of enrolled patients), including 351 patients without CKD (corresponding to 24%) and 350 patients with CKD (70% of CKD patients; p<0.001 *vs.* patients without CKD). The most important results of this study are:

- according to the ESC 2020 recommended baseline,[117] a hs-cTnT threshold of ≥52 ng/L provided substantially lower performance for ruling-in AMI in patients with CKD [Positive predicted value (PPV) 36%] compared to those without CKD (PPV 61%), with even lower performance in those with advanced CKD (PPV 25%);[192]
- higher baseline hs-cTnT thresholds significantly improved performance for ruling-in AMI in CKD patients with PPV reaching 80% in those with concentrations >300 ng/L;[194]
- serial 2 h hs-cTnT measurements improved the diagnostic accuracy for ruling-in AMI in CKD patients;[194]

• higher 2 h hs-cTnT thresholds (>20 ng/L and >30 ng/L) provided excellent performance in ruling-in AMI in these patients, including those with advanced CKD.[194]

Overall, the results of this study suggest that diagnostic performance of standard baseline and serial 2 h hs-cTnT thresholds to rule-in AMI is suboptimal in CKD patients. However, diagnostic performance of standard baseline and serial 2 h hs-cTnT thresholds can be significantly improved if higher baseline thresholds and delta values are taken into account.[194] Furthermore, the results of this study suggest that CKD patients have poor long-term outcomes, particularly those with type 2 AMI and myocardial injury.[194] Considering the outcome evaluation at 2-years, a poor outcome was related to older age, higher comorbidity burden (especially heart failure), and lack of guideline-directed evaluations and therapies.[194] In particular, CKD patients had higher post-discharge MACE at 2 years with a concomitant index diagnosis of acute MI (57% *vs.* 35%, p=0.01), type 2 MI (60% *vs.* 37%, p=0.03), and myocardial injury (55% *vs.* 41%, p<0.001) compared to those without CKD.[194]

More specifically, Bjurman *et al.*[195] examined the effects of renal function and a medical record of cardiovascular disease on levels of several biomarkers, including: hs-cTnI, hs-TnT, N-Terminal pro-Brain Natriuretic Peptide (NT-proBNP), copeptin, human fatty acid-binding protein (hFABP), and cystatin C. It is important to note that in this study an accurate estimation of GFR was obtained using Cr51-EDTA or iohexol clearance in 460 patients (56% males, median age 58, IQR 45-65 years), prospectively referred at the Department of Clinical Physiology (Sahlgrenska University Hospital, Gothenburg, Sweden).[195] The patients were divided based on the presence (N.=223) or absence (N.=237) of cardiovascular disease. Authors reported that hs-cTnT levels correlated more strongly to measured GFR and increased more at low GFR compared to hs-cTnI.[195] However, even if the results of this study were not able to clarify to which extent the increased biomarker production or decreased kidney clearance contributed to the increases in hs-cTnI and hs-cTnT levels observed among patients with a low GFR, because the extent of the elevation of cardiac markers at low renal function is highly variable.[195]

Take-home messages

• Several authors underlined the fundamental role played by renal function in influencing circulating levels cTnI and cTnT in both healthy individuals and in patients with cardiovascular diseases and/or other comorbidities, particularly in acute and CKD;[179-188, 195]

• from an analytical point of view, further validation studies are needed to develop standardized protocols for hs-cTnI and hs-cTnT assays able to accurately measure these biomarkers in urine samples, collected from both healthy subjects and patients with cardiovascular diseases and/or other comorbidities;

• from a clinical point of view, it is well established that cardiovascular disease is a major complication of CKD and remains a leading cause of mortality in this patient population, which experiences high rates of recurrent hospitalizations, debilitating symptoms, and reduced health-related quality of life;[186-190]

• in particular, data from multiple epidemiologic and cohort studies as well as intervention studies suggest that CKD is an independent risk factor for cardiovascular disease and that patients with advanced CKD stages (CKD stages 4–5) are at significantly higher risk of cardiovascular disease, being sudden cardiac death the most relevant single cause of death in chronic dialysis patients;[186-188, 193-195]

• nevertheless, the diagnosis of AMI in patients with CKD remains difficult as these patients often exhibit chronically elevated hs-cTnI and hs-cTnT concentrations.[182-188, 193-195]

Advancements and challenges in cardiac troponin measurement

Future trends in analytical characteristics and performance of hs-cTnI and hs-cTnT methods

Despite the most recent methodological advances, some challenges still remain to improve the assay specificity of hs-cTnI and hs-cTnT methods.[7-9, 21-23] In particular, the available hs-cTnI and hs-cTnT methods are unable to distinguish between lower and higher molecular forms of circulating biomarker in blood of healthy subjects (especially after strenuous physical exercise) or patients with cardiac or extra cardiac diseases.[7, 9, 26, 27, 43, 45-47, 49, 52-54, 196, 197] Considering the Fourth Universal Definition of Myocardial Infarction,[1] the results reported by several experimental and clinical studies have clearly indicated that the possibility

to discriminate between low and higher molecular forms of circulating cardiac troponins may be a decisive finding able to discriminate between reversible or irreversible myocardial injury.[5-9, 23, 36-43, 52-54, 128] Indeed, increased values above the 99th percentile URL value of circulating hs-cTnI and hs-cTnT with high molecular form should be detected only in presence of an irreversible damage of myocardial tissue.[35-43] In particular, some recent studies have reported that heavily truncated small fragments of cTnT seem to be responsible for the increased hs-cTnT circulating levels in patients with renal failure and healthy subjects after strenuous exercise, whereas intact and long forms of cTnT have been detected early after AMI.[41, 49, 54, 197-200] Conversely, the long forms of cTnT (*i.e.*, the intact and long fragments with MW of about 28 to 40 kDa) are most prominent in early-presenting AMI patients.[37, 42, 46, 49, 54, 197, 198]

In 2022, Airaksinen *et al.*[197] assessed the ability of a novel immunoassay targeted for long cTnT forms (including intact and mildly truncated cTnT) to discriminate biomarker elevations caused by type 1 AMI and/or end-stage renal disease (ESRD). Authors developed a novel, simple time-resolved, fluorescence-based sandwich immunoassay able to measure only the longer forms of cTnT, that are intact and only mildly truncated. The capture antibody (HyTest monoclonal antibody 7E7) binds to an epitope at the C-terminal part of cTnT (amino acid residues 223-242 according to UniProt entry P45370-6) and three tracer antibodies (Hytest monoclonal antibodies 7G7, 329cc, and 1C11) bind different areas of the central part of cTnT (amino acid residues 67-86, 119-138, 174-190, respectively). This assay targets epitopes very close to each other in the middle of the central part of cTnT (amino acid residues 125-130 and 136-147) and it can detect intact, mildly truncated and also heavily truncated short forms of cTnT.[197] Authors report that this new assay is able to measure the total cTnT concentration and also to assess the ratio of long cTnT forms/total cTnT in the samples as a measure of protein fragmentation.[197] The analytical performance of this new cTnT assay can vary in relation to the specific cTnT forms detected: LoD from 3 to 11 ng/L, the LoQ (10% coefficient of variation) from 13 to 25 ng/L, and the measuring range high end from 10,000 to 50,000 ng/L.[197] The authors collected heparin plasma samples from 46 patients with NSTEMI, 71 patients with STEMI, and 40 patients with ESRD on maintenance hemodialysis.[197] The most important results of this study were:

- total cTnT levels did not differ between the patients with NSTEMI and those with ESRD, but were higher in patients with STEMI;
- the ratio of long/total cTnT was higher in both MI groups than in patients with ESRD;
- the ratio was also higher in patients with a ≤24-hour sample delay after symptom onset than in those with longer delays both in the NSTEMI (median, 0.29 *vs.* 0.15) and STEMI groups (median, 0.62 *vs.* 0.22; P<0.001 for both) resulting in a more pronounced difference in troponin composition compared to ESRD (median ratio, 0.04) in the early phase of the acute cardiac diseases. The authors concluded that this new immunoassay approach is much simpler and more sensitive than the laboratory methods previously adopted to study cTnT fragmentation, that require some preliminary and complex chromatographic purification of the samples before the hs-cTnT measurement.[197]

More recently, Salonen *et al.*[198] have developed a novel immunoassay specific for long forms of cTnT. An immunoassay specific for the biomarker long forms requires a lower LoD in comparison to the standard hs-cTnT assay, because the long forms represent only a fraction of the total circulating forms of the biomarker. This method utilizes upconverting nanoparticles (UCNPs) as reporters, which are able to minimize the autofluorescence background in immunoassay systems, significantly improving the assay performance. Indeed, this assay shows a limit of blank (LoB) of 0.15 ng/L, a LoD of 0.40 ng/L and a LoQ (at 10% CV) of 1.79 ng/L.[198] Li-heparin and EDTA plasma, but not serum, were found to be suitable sample matrixes for the assay. Using the ROC analysis, the troponin ratio (long/total cTnT) evaluated with this novel assay, showed excellent discrimination between NSTEMI and ESRD with an AUC value of 0.986 (95% CI 0.967-1.000).[198] A significant limitation of the long cTnT method is that the assay in its current form is not suitable for clinical practice in emergency setting, as it requires manual operative procedure and 5 h of the total analytical time.[198]

According to several recent observations and experimental results,[9, 53, 54, 196-200] the analysis of troponin fragments can represent a new fundamental new chapter in the history of cardiac troponin assay. Until now, the fragmentation of cardiac troponins *in vivo* has been investigated only to a limited extent, because the lack of specificity of hs-cTnI and hs-cTnT methods so far available in recognizing the different forms of biomarkers.[8, 9, 17, 23, 38-40, 42, 46, 48, 49, 52-54, 199] From a pathophysiological point of view, the availability of some specific methods for cardiac troponin fragment analysis could improve our ability to distinguish the mechanisms of cardiac troponins release from the damaged cardiomyocytes so that distinctions can be made between direct myocardial injury, sup-

ply–demand abnormality, and acute coronary occlusion in patients admitted to ED with acute thoracic pain.[36, 39, 40, 199] Moreover, considering the case of the extreme exercise in apparently healthy subjects or athletes, the frequently observed increase in circulating hs-cTnI and hs-cTnT levels is predominantly due to a release of smaller (and shorter) forms of cardiac troponins from cardiomyocytes.[7, 41, 52-54] Accordingly, the analysis of troponin fragments using some specific and sensitive cTnI and cTnT immunoassays could be useful to exclude the presence of an irreversible myocardial damage after strenuous physical exercise.

As also recently observed by other authors,[199, 200] further work in the development of sensitive and specific immunoassays for the analysis of troponin fragmentation is clearly required, since these novel methods could have the potential to significantly improve the diagnostic evaluation related to the variations of cTnI and cTnT levels in healthy subjects and in athletes after strenuous physical exercise and in patients with cardiovascular diseases.

Diagnostic, pathophysiological, and clinical perspectives of cardiac troponin assay

The measurement of hs-cTnI and hs-cTnT concentrations is specifically recommended by all international guidelines for the diagnosis of NSTEMI-ACS.[1-9] Moreover, the results of a recently published meta-analysis confirmed that hs-cTnI and hs-cTnT methods do not show significant difference in prediction of multiple cardiovascular endpoints in the community dwelling adults.[201] It is also well known that increased hs-cTnI and hs-cTnT levels over the 99th percentile URL value, can be found virtually in all acute or chronic clinical cardiac conditions, including: arrhythmias, cardiac ablation, cardiac contusion, infiltrative cardiac disorders, defibrillation shock, cardiac failure, and congenital heart diseases.[1-18] Furthermore, increased hs-cTnI and hs-cTnT levels over the 99th percentile URL value, have been commonly found in some patients with non-cardiac conditions, including: pulmonary embolism, impaired renal disease, sepsis/systemic inflammatory response syndrome and critically ill patient, rhabdomyolysis, burns, drug toxicity and stroke.[1-12]

In particular, the evaluation of cTnI and cTnT variations in patients with acute and CKD deserves a further and deeper discussion. In 2023, an authoritative review from the European Renal and Cardiovascular Medicine Working Group of the European Renal Association[185] pointed out that cardiovascular complications are the most common causes of death in patients with kidney failure (stage G5), who are maintained on regular dialysis treatment. Moreover, the data reported indicate that most patients with progressive CKD die before reaching kidney failure, because the high death rate attributable to cardiovascular disease.[185] Accordingly, the authors conclude that concerted efforts are being made by major scientific societies to advance basic and clinical research on cardiovascular disease in CKD patients.[185] Even if the cited review discusses in detail the risk factors for cardiovascular diseases in CKD patients, the role of specific cardiac biomarkers in assessing the cardiovascular risk in CKD patients is not evaluated.[185] Conversely, the Italian Study Group of Cardiac Biomarkers believes that the measurement of hs-cTnI and hs-cTnT levels in CKD patients can play a relevant clinical role, according to the evidences reported in the literature.[182, 183, 190-194]

A significant inverse association between reduced eGFR values (considered as an indicator of renal function) and increased circulating hs-cTnI and hs-cTnT levels has been frequently reported both in the general population and in CKD patients.[15, 172, 182, 190, 191] This inverse relationship can be extended throughout all the eGFR range (*i.e.*, from norma1 to kidney failure).[15] Moreover, the results of several experimental and clinical studies indicate that hs-cTnI and hs-cTnT levels can be influenced by renal function in CKD patients.[10-15, 17, 157, 168, 180, 183] In particular, the results of a very large population study suggest that moderate reductions in eGFR (30-59 mL/min/1.73 m^2) correspond to a median cTnT value above the 14 ng/L of the upper reference limit.[15] However, the results of these studies were not able to provide an accurate estimation whether the increased hs-cTnI and hs-cTnT circulating levels are due to cardiac damage or reduced renal clearance, respectively. Indeed, hs-cTnI and hs-cTnT levels are often increased in CKD patients, even in an absence of specific cardiac symptoms.[183] Some authors believe that the increased hs-cTnI and hs-cTnT circulating levels typically reflect the extent of underlying cardiovascular disease rather than an impairment of renal clearance.[180]

It is well known that there is a substantial overlap in comorbidities between cardiovascular and CKD, such as diabetes and hypertension.[180-186] From this point of view, the progression to renal failure can be considered as a marker of disease severity driven by these same comorbidities, representing a link of common pathophysiology mechanism for progression toward ESRD and cardiomyopathy.[180] This hypothesis is also well in accordance with the large body of evidence derived from multiple epidemiologic and clinical studies reporting that CKD is an independent risk factor for cardiovascular disease.[180, 186-188, 191, 192] On

the other hand, patients with advanced CKD stages (CKD stages 4-5) are at significantly higher risk of cardiovascular disease, being the sudden cardiac death the largest single cause of death in chronic dialysis patients.[184-186, 201, 202]

In 2021, Sun *et al.*[203] demonstrate in 181 stable maintenance hemodialysis patients that hs-cTnT is a powerful independent predictor of cardiovascular outcome and all-cause mortality. The Authors also evaluated the increased discriminative value associated with the addition of echocardiographic parameters and hs-cTnT using net reclassification improvement (NRI) and integrated discrimination improvement (IDI). During follow-up (median follow-up = 31, IQR: 21-33 months), 37 patients died, 84 patients suffered one or more cardiovascular events, and 78 patients developed 4-point MACE.[203] The hs-cTnT measurement was associated with improvements in reclassification for fatal or non-fatal cardiovascular events (NRI=44.6% [15.9-74.3%], IDI=15.9% [5.7-31.0%], all p<0.001), all-cause mortality (NRI=35.5% [10.1-50.2%], p<0.001, IDI=4.4% [1.3-8.5%], p=0.005), and MACE (NRI=47.2% [16.1-64.9%], p<0.001, IDI=16.9% [5.5-37.3%], p=0.005). Interestingly, the additional use of echocardiography to improve the risk stratification was not supported by the results of this study.[203]

More recently, the results of a meta-analysis,[201] including 23 articles (related to a 36 cohort studies), have confirmed that both hs-cTnI and hs-cTnT had high sensitivity in predicting cardiovascular risk in CKD patients (*i.e.*, 24 studies for all-cause mortality and 12 studies for cardiovascular mortality). In particular, for each 10 ng/L increase in hs-cTnT and hs-cTnI values, the relative risk (RR) increased by 14% (RR=1.14, 95% CI 1.10-1.18) and 19% (RR=1.19, 95% CI 1.09-1.31) for all-cause mortality, 25% (RR=1.25, 95% CI 1.13-1.38) and 19% (RR=1.19, 95% CI 1.10-1.29) for cardiovascular mortality, respectively.[201] Interestingly, the heterogeneity between patients on dialysis or non-dialysis was not statistically significant, suggesting that dose-response association between hs-cTnI (*i.e.*, curvilinear relationship) and hs-TnT (*i.e.*, linear relationship) and all-cause mortality seems stable for all CKD stages.[201]

Considering the impressive amounts of evidences reported in several experimental and clinical studies discussed in this document,[180-186, 188-195, 201-204] the Italian Study Group of Cardiac Biomarkers believes that an important role for the evaluation of cardiovascular risk in CKD patients should be recognized utilizing the measurement of hs-cTnI and hs-cTnT levels and that message should be also shared by all expert documents and international guidelines to be published in the upcoming future.

Moreover, as observed in the previous paragraph of this document, the development of new sensitive and specific immunoassays for the analysis of troponin fragmentation could improve also our knowledge about the pathophysiological mechanisms related to the increase of hs-cTnI and hs-cTnT frequently found in CKD patients.[41, 49, 166-168, 182-185, 193, 194, 197] In 2022, Airaksinen *et al.*[197] using a novel, simple time-resolved, fluorescence-based sandwich immunoassay to measure the long forms of cTnT, reported that total cTnT did not differ between the 46 patients with NSTEMI and 46 patients with ESRD, but was higher in 71 patients with STEMI. Overall, the results of this study[197] confirm the previous data of other experimental clinical studies reporting that the intact and mildly truncated cTnT forms are typical of patients with type 1 AMI in the early hours of myocardial damage, whereas small fragments are the main components of cTnT elevation in ESRD patients.[41, 49, 166-168] Of course, the results of this preliminary report should be confirmed by results of other clinical studies including a larger population of control healtly subjects and CKD patients with or without MI.

Conclusions

- Even if the hs-cTnI and hs-cTnT measurement is specifically recommended by all international guidelines for the diagnosis of NSTEMI-ACS,[1-9] increased hs-cTnI and hs-cTnT levels can be found virtually in all acute or chronic clinical cardiac conditions;[1, 6, 7, 9, 17]
- an inverse association between eGFR values (considered as an indicator of renal function) and circulating hs-cTnI and hs-cTnT levels has been frequently reported both in general population and in CKD patients;[10-15, 17, 157, 168, 180, 193, 194]
- in CKD patients, increased hs-cTnI and hs-cTnT circulating levels reflect the extent of underlying cardiovascular disease rather than an impairment of renal clearance;[180]
- the results of recent published studies confirmed that both hs-cTnI and hs-cTnT had high sensitivity in predicting cardiovascular risk in CKD patients for both all-cause mortality and specific cardiovascular mortality;[201-204]
- taking into account the most recently available evidences,[201-205] hs-cTnI and hs-cTnT measurement plays an important role in evaluating cardiovascular risk in CKD patients;
- considering future trends in analytical characteristics and performance of cTn methods it is important to note that the very recent development of novel sensitive and

specific immunoassays for the analysis of troponin fragmentation could have the potential to significantly improve the diagnostic evaluation related to the variations of cTn levels in healthy subjects and athletes after strenuous physical exercise as also in patients with cardiovascular and/or kidney diseases.[196-200]

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