HIGH-SENSITIVITY TROPONIN: A NEW TOOL FOR PATHOPHYSIOLOGICAL INVESTIGATION AND CLINICAL PRACTICE

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1. Abstract

At the dawn of the new century, the advent of more specific myocardial tissue markers, such as cardiac troponin I (cTnI) and T (cTnT), has led to a new definition of acute myocardial infarction (AMI) by international guidelines. If we accept the concept that AMI is the portion of acutely necrotic myocardial tissue (irrespective of size), some patients previously diagnosed with severe angina may be currently considered to present minimal (even microscopic) quantities of myocardial necrosis. Although increased cTnI or cTnT values always indicate myocardial tissue damage, a positive test is not able to identify the mechanism responsible for that cardiac damage (which could be not due to ischemia). New cTnI and cTnT immunoassays with increased analytical sensitivity may increase "false positive" results in patients with cardiovascular disease, especially those with advanced age, heart failure (HF), severe comorbidities (such as chronic renal insufficiency), or assuming potential cardiotoxic drugs. Hence, it may be not clear for most patients and physicians whether high-sensitivity cTnI and cTnT methods will lead to more clarity or confusion. The aim of this review is to update the present knowledge in the field of cTnI and cTnT with particular attention on the impact of immunoassays with increased analytical sensitivity on both laboratory and clinical practice.

2. Background and Aim

About 25 years ago, few diagnostic tests were available for clinical practice and considered useful in the assessment of cardiac necrosis, such as those measuring the total enzymatic activity of creatine kinase (CK) and lactate dehydrogenase. Unfortunately, those methods were characterized by low sensitivity and specificity for cardiac damage. Some immunoassay methods for structural proteins and cardiac isoenzymes, such as CK-MB isoenzyme and myoglobin, were then developed (Fig. 1). These markers showed an increased sensitivity, but only a relative specificity for cardiac disease, because these proteins are also present in the skeletal tissue.

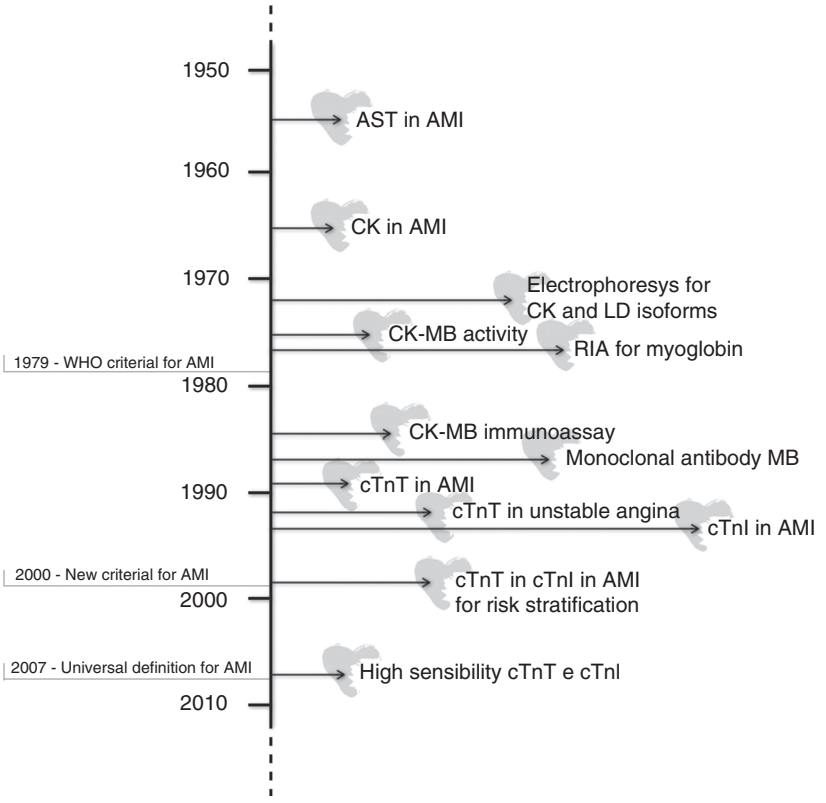

FIG. 1. Brief history of cardiac marker for myocardial damage.

At the dawn of the new century, the advent of more specific myocardial tissue markers, such as cardiac troponin I (cTnI) and T (cTnT), ledch to the new definition of AMI by international guidelines [1, 2]. If we accept the concept that AMI is the portion of the myocardial tissue (despite size) with acute necrosis due to myocardial ischemia, several patients, previously diagnosed to have a severe angina, should be currently considered to present minimal (even microscopic) quantities of myocardial necrosis [1, 2]. As a result, the new definition of myocardial infarction has had a high impact on both laboratory and clinical practice [3–8]. The clinical application of international guidelines [1] generated main social/economical effects, leading to a 25–55% increment of diagnosed AMI [3–5].

Although increased cTnI or cTnT values always indicate myocardial tissue damage, a positive test is unable to identify the mechanism responsible for

that cardiac damage (which could be not due to ischemia). The advent of the new cTnI and cTnT immunoassays with increased analytical sensitivity may increase "false positive" AMI results in patients with cardiovascular disease, especially those with advanced age, HF, severe comorbidities (such as chronic renal insufficiency) or being treated with potential cardiotoxic drugs [3–5]. Hence, it may be not clear for most patients and physicians whether the new high-sensitivity cTnI and cTnT methods will lead to more clarity or confusion.

To clarify these important clinical issues, a computerized literature search on National Library of Medicine (i.e., PubMed access to MEDLINE citations, http://www3.ncbi.nlm.nih.gov/PubMed/) was performed in June 2009 using keywords such as "troponin assays" (>7000 articles) and "high-sensitive troponin assays" (~180 articles). The aim of this review is to update the present knowledge of cTnI and cTnT with particular attention to the impact of these new immunoassays with increased analytical sensitivity (i.e., the so-called high-sensitivity cTnI and cTnT immunoassay methods) on both laboratory and clinical practice.

3. Introduction: Troponin Framework Within Myocardial Cells and Release Kinetics After Myocardial Damage

Troponin is a complex of three integrated proteins essential for both muscle contraction and relaxation, regulated by intracellular calcium concentration [9]. The troponin complex plays a fundamental role in the contraction of both cardiac and skeletal muscles, but not of smooth muscles. This complex interacts with two key molecules of the contractile process, the thin actin and the thick myosin filaments. Troponin is linked to the tropomyosin protein and is positioned among actin filaments within the muscle tissue. The three complex subunits, troponin C (TnC), troponin I (TnI), and troponin T (TnT), share different physiologic properties. TnT binds the troponin group to tropomyosin, forming a troponin–tropomyosin complex, which is responsible for contraction. TnI binds to actin, secures the troponin–tropomyosin complex, and leads to muscle relaxation by interrupting the actin–myosin linkage. TnC binds to calcium ions producing a structural change in TnI, in order to interrupt relaxation and to begin the contraction cycle.

Skeletal isoforms of TnT and TnI are replaced by cardiac-specific isoforms during fetal development of the human heart. At the end of the last century (Fig. 1), specific immunoassays for identifying cardiac muscle damage were

developed using antibodies to cTnI and cTnT. These assays were specific for identifying cardiac muscle damage and were free from interferences due to the presence of skeletal muscle isoforms [10]. First generation cTnT assays were, however, susceptible to false positivity due to cross-reactivity with skeletal TnT antibody [10]. Second generation immunoassay methods, designed using more highly specific antibodies, solved the interference problem with skeletal muscle isoforms and showed comparable results with cTnI assays [11–13]. Substantial data exist today that conclusively demonstrate that methods that rely on cTnI and cTnT detection share absolute specificity for myocardial damage.

Cardiac troponins appear in the serum relatively early following onset of AMI (2–10 h), peak at 12–48 h, and remain abnormal for 4–14 days (cTnI 5–10 days and cTnT 5–14 days) [11–13]. These release kinetics can be accounted for by examining the distribution of the proteins within the myocardial cell. The great majority of both cTnI and cTnT is bound to the myofibril (94–97%), and only a relatively small amount ($\sim$3% for cTnI and 6% for cTnT) free in the cytoplasm [11, 14]. Following cardiac cell injury and immediate release of the free cytoplasmic pool, there is a slow, but continuous and prolonged release of troponins presumably from myofibril-bound proteins [11, 14]. It is unclear, however, whether this early releasable troponin pool is actually free in the cytoplasm or loosely bound to myofilaments.

4. Impact of the New Definition of Myocardial Infarction on Laboratory Practice and Instrumentation: The Need for High-Sensitivity cTnI and cTnT Methods

4.1. Quality Specifications for cTnI and cTnT Immunoassays

According to the new definition of AMI [1, 2], cardiac-specific troponins (cTnI and cTnT) are the preferred biomarkers, and if available, they should be measured in all patients with typical chest pain. An increase of cTnI or cTnT levels over the 99th percentile upper reference limit (99th URL) (cut off value) should be considered clinically relevant. Furthermore, it is recommended that cTnI and cTnT values corresponding to the 99th URL should be measured with an imprecision, or coefficient of variation (CV) $\leq$10% [1, 2]. Finally, it has been suggested by international guidelines [1, 2] and quality specifications [6] that each laboratory independently confirm reference intervals, although assay standardization is preferable [4, 6, 7].

The first important analytical issue is epitope location on the troponin molecule. It is important to note that the amino- and carboxy-terminal ends are more susceptible to proteolysis and this degradation may be related to the degree of tissue ischemia. Interestingly, these modified "partially degraded" products, not intact cTnI, were specifically detected in effluents from severely ischemic hearts [11]. International guidelines [6] for immunoassay development have recommended that the epitope should be identified and located within a stable region of the cTnI molecule. Furthermore, specific relative responses are required for cTnI forms. These include free cTnI, the I–C binary complex, the T–I–C ternary complex, and oxidized, reduced, and phosphorylated isoforms of the three cTnI forms [6]. cTnI and cTnt can be determined by a number of commercial immunoassays with different epitope-specific antibodies. As such, it can be expected that differences in assay response to the various troponin forms probably detect slightly different patient populations depending on the nature and timing of cardiac troponin release [11, 13, 14]. These complications, in addition to differences in assay generation, create a substantive problem for clinical and laboratory interpretation of test results.

The second important analytical issue is specificity of troponin antibodies. Apart from the cTnT method, which is offered by one patent-protected vendor, there are more than 20 cTnI immunoassays commercially available [14, 15]. It can be safely assumed that antibodies in these different assays do not bind all to the same epitope and therefore they measure different cTnI forms. In addition, cTnI assays vary with respect to the antigen used for calibration, antibody type itself, and indicator molecule. Detection of antigen–antibody complexes also vary and may involve spectrophotometric, fluorescent, chemiluminescent, or electrochemical methods. Consequently, different TnI assays do not produce equivalent concentration results [4–8] and comparison of absolute troponin concentration should not be made [14]. Indeed, numerous manufacturers have developed their own 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 [4–8]. Unfortunately, standardization of cTnI methods, despite continuous solicitation and recommendations [4, 6–8], has been difficult to achieve and remains in progress [14, 15].

Many, or even most, commercially available cTnI and cTnT methods do not actually report the 99th URL value, nor achieve the precision (10% CV) required for assay reproducibility at the cutoff [4–8]. Increased assay precision and improved standardization is mandatory in order to achieve common reference and decision limits for troponin immunoassays in accordance with international guidelines and quality specifications [1, 2, 6].

4.2. The Development of High-Sensitivity Immunoassays for cTnI and cTnT Measurement

The European Society of Cardiology (ESC), the American College of Cardiology Foundation (ACCF), the American Heart Association (AHA), and the World Heart Federation (WHF) now recommend a single cTnI and cTnT decision cut-point for the diagnosis of myocardial infarction in patients presenting with suspected myocardial necrosis correspond to the 99th percentile upper reference limit (99th URL) [2]. This very low cut off concentration, however, creates a significant problem because most assays lack the analytical sensitivity to consistently measure troponin in the blood of apparently healthy individuals. This results in a high proportion of reference population values below the limit of detection for most methods. As such, the 99th URL cannot be ascertained with any acceptable degree of analytic certainty or basis [15, 16]. Furthermore, the new definition of AMI [1, 2] that specifically requires assay precision $\leq 10\%$ CV for the 99th percentile of the reference population, remains a difficult challenge for manufacturers of commercial cTnI and cTnT immunoassays. In fact, following establishment of the new AMI definition [1], no commercial immunoassay was able to fulfill this recommendation [7, 8].

The development of more sensitive and better precision assays should permit more reliable estimation of very low cTnI and cTnT concentration. It is likely that significant improvement in troponin assay sensitivity is required to reproducibly measure near or below the ng/L concentration where reference values may be Gaussian-distributed [5, 14]. As a result of this challenge, next generation of cTnI and cTnT assays have been recently developed to improve the analytical performance and standardization [17–29]. It is noteworthy that some of these new methods are characterized by improved low-end analytical sensitivity and precision, which should increase precision at the cutoff (99th percentile of the reference population) to about 10% or even better (Table 1).

TABLE 1
Detection Limit, Analytical Sensitivity, and 99th URL of Some Highly Sensitive Immunoassay Methods for cTnI and cTnT

Method	DL (ng/L)	10% CV (ng/L)	99th URL (ng/L)	Ratio	References
cTnI Assay					
Ultra ADVIA Centaur	6	57	72	0.8	21, 28, 29
Singulex Erenna	0.2	0.91	9	0.1	24
Ultra Accu TnI	6	14	40	0.35	18, 25, 45
cTnT Assay					
Elecsys hs TnT	2	12	14	0.85	20, 22, 23

DL, Detection limit.

4.3. Definition of Highly (Ultra) Sensitive Immunoassay for cTnI and cTnT

An important issue in the development as well as in the practical use of highly sensitive cTnI and cTnT immunoassays is the appropriate definition of assay sensitivity. This definition directly impacts two aspects of assay performance: limit of detection and assay precision [15]. Accurate discrimination of "minor" myocardial damage versus analytical noise requires assays with excellent limit of detection and a high precision at low troponin concentration. New generation cTnI and cTnT immunoassays have been characterized by a limit of detection at the picogram or subpicogram level (Table 1). A simple calculation may better explain the impact of increased analytical sensitivity in clinical practice. For example, highly sensitive cTnI and cTnT methods have a limit of detection <10 pg/mL (Table 1). Cardiomyocytes contain ~ 70 mg cTnI per gram myocardial tissue [30], ~ 10 pg cTnI is contained in 1 mg myocardial tissue [31]. As such, it is conceivable that necrotic damage to 10–50 mg of myocardial tissue should be detectable by highly sensitive troponin methods.

There is a lack of consensus regarding the best method to assess immunoassay sensitivity at very low analyte concentration [15]. The limit of detection has been defined by the Clinical and Laboratory Standards Institute as the lowest amount of analyte in a biological sample that can be reliably detected by a given analytical procedure [32]. The limit of detection for cTnI and cTnT is usually estimated as the protein concentration that corresponds to a signal 2 or 3 standard deviations (SD) above the mean of at least 20 replicates for a sample absent in troponin (zero calibrator). This calculated value should be considered as the limit of blank, which is defined as the highest measurement result likely to be observed (with a stated probability) for a sample that contains no troponin, rather that the true assay limit of detection [15, 32]. Thus, the major difference in estimating assay limit of detection and limit of blank is the sample type used for measurement. Zero calibrators, assay diluents, or serum troponin-free (recommended) are only useful for determination of limit of blank, while in order to have an adequate estimation of the immunoassay detection limit, serum samples containing cTnI or cTnI concentrations in the range from the blank value to fourfold the blank value should be used [15, 32]. Therefore, limit of detection values reported in the literature are generally lower than those obtained using the recommended experimental procedure [32].

From a clinical point of view, the most important analytical characteristic should be the limit of quantification [15, 32], also called functional sensitivity [33]. This quantity is defined as the lowest amount of analyte (cTnI or cTnT) that can be quantitatively measured with stated acceptable precision and bias (i.e., measurement uncertainty) [34]. As previously mentioned, international guidelines [1, 2] specifically require an assay precision $\leq 10\%$ CV for the 99th

percentile of the reference population. It is important to note that this degree of precision ($\leq$10% CV) is slightly better than an optimal total error goal (12% CV) as suggested by other authors considering the biologic variation of cTnI [14, 35]. According to international guidelines and quality specifications [1, 2, 8, 36], it is conceivable that an immunoassay for the measurement of cTnI or cTnT should be defined to be highly (or ultra) sensitive if it is capable of measuring the 99th percentile of the reference population with an total error $<$10% CV (10% CV concentration to 99th percentile limit ratio $<$1). However, there are some analytic and clinical problems concerning this definition.

First, there is lack of consensus in the literature about the best method to assess and report precision data [14]. Manufacturer assay package inserts often report only precision based on within-run or between-day evaluation of samples with cTn concentrations much higher than the AMI cutoff. Furthermore, these data do not usually include lot-to-lot and machine-to-machine (interinstrument) variability [14]. As such, cTnI and cTnT assay variability determined within the clinical laboratory is frequently higher than those quoted by the manufacturer. For example, the ADVIA Siemens TnI-Ultra^{TM} concentration at 10% CV determined across two hospital sites and five analyzers using three reagent lots was 0.10 μg/L [26] compared with 0.03 μg/L per package insert and 0.05–0.07 μg/L from literature studies [27–29, 37].

Second, some troponin values, measured in the reference population, may be still below the analytical sensitivity of the new generation immunoassay methods [28–39]. This suggests that the precision at low troponin values of these methods should be further improved in order to measure the protein concentration in each plasma sample of all healthy subjects. In consideration of these findings, Apple [39] recently suggested to divide the new cTnI and cTnT methods into four levels, according to the percentage of measurable normal values below the 99th percentile: level 1 (contemporary) $<$50%; level 2 (first generation with high sensitivity) from 50% to $<$75%; level 3 (second generation with high sensitivity) from 75% to $<$95%; level 4 (third generation with high sensitivity) $\geq$ 95%. However, the fraction of measurable normal values may strongly depend on some demographic characteristics (i.e., gender, age, and myocardial ventricular mass) of the reference population studied [16, 21, 28]. These issues will be discussed in the following sections.

5. The Impact of High-Sensitivity cTnI and cTnT Methods on Clinical Practice

International guidelines [1, 2] recommend the measurement of cardiac damage markers (usually cTnI or cTnT) in each patient with suspected acute coronary syndrome (ACS). From a clinical point of view, only cardiac

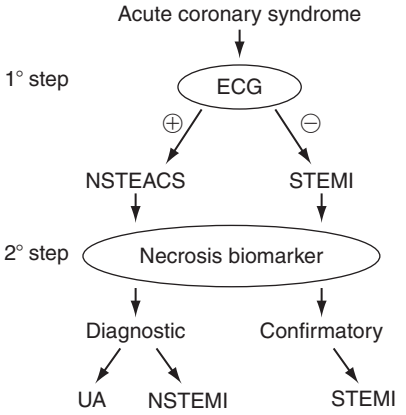

FIG. 2. Differential diagnosis of acute coronary syndromes according to consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction [1].

troponin measurement is able to distinguish patients with unstable angina (UA) from those with non-ST-segment elevation myocardial infarction (NSTEMI) (Fig. 2) [1, 2]. However, increased cTnI or cTnT values alone are unable to indicate the pathophysiological mechanism underlying the detected myocardial damage, which may be unrelated to ischemia. Therefore, an increased marker value, without clinical indication of myocardial ischemia, should prompt a search for other causes of cardiac damage (Table 2). On the other hand, there is clear evidence that any amount of detectable cardiac troponin is associated with an increased risk of new adverse cardiac events in patients with ACS, with no record of a threshold below which troponin elevation is harmless and meaningless for prognostic stratification [40–43]. These data suggest that there may be some unresolved problems in the definition of decisional (i.e., cut off) values for differential diagnosis in ACS, as well as, risk stratification of patients with cardiovascular diseases. These issues will be discussed and clarified in the following sections.

5.1. The Problem of Reliable Definition and Accurate Estimation of the 99th Percentile Upper Reference Limit: Can Reference Values Be Affected by any Characteristics of the Reference Population?

In their Universal Definition of AMI, the ESC/ACCF/AHA/WHF Task Force for the Redefinition of AMI recommended, as criteria for nonprocedure-related AMI, the evidence of increased or decreased cTnI or

TABLE 2
THE MOST FREQUENT CLINICAL CONDITIONS IN WHICH THE CIRCULATING LEVELS OF CARDIAC TROPONINS ARE INCREASED, WITHOUT OVERT CORONARY ARTERY DISEASE

- Myocarditis/pericarditis
- Congestive heart failure
- Systemic arterial hypertension
- Systemic arterial hypotension (especially if associated with cardiac arrythmias)
- Cardiac surgery or catheterization (e.g., ablation)
- Critically ill patients
- Hypothyroidism
- Cardiac trauma
- Myocardial toxicity from cancer therapy
- Pulmonary embolism
- Episode rejection of a cardiac transplant
- Postoperative noncardiac surgery
- Chronic renal failure
- Amyloidosis
- Sepsis

cTnT with one or more values above the 99th URL, found in a clinical setting suggestive of myocardial ischemia, together with either clinical symptoms, new ischemic ECG changes, or imaging findings of new loss of myocardium [2]. According to this definition, a reliable estimation of the 99th URL assumed a central role in the clinical diagnosis of AMI. In addition to assay sensitivity, the main factor that may influence the 99th URL estimation is the selection of the reference population, including number, type, age, and gender of individuals enrolled in the study [17, 19, 21, 28, 38, 44–46]. Finally, the matrix of the sample employed (serum or plasma) for this specific evaluation may also affect results [15].

The sample size is an important factor to take into account for the 99th URL estimation. International guidelines recommend a minimum of 120 reference individuals per group for appropriate statistical determination of reference limits [47]. However, a sample size of at least 300 individuals is required to reach the 95% probability, that at least 99% of the population will fall below the highest observed analyte value [48] (Table 3). Under these conditions, the uncertainty in defining true 99th URL is high because the cut off concentration is approximately equal to the individual having the third highest cTnI or cTnT concentration. Thus, if three more apparently healthy individuals with somewhat increased troponin concentrations were included in the reference group, the calculated 99th URL would be substantially changed [15].

Age- and gender-dependent differences may also have significant clinical relevance on the 99th URL estimation. This has been demonstrated in recent

TABLE 3
Sample Sizes and Associated Tolerance Levels for Reporting 99th Percentile Based on the Largest Observed Value (According to Reference 48)

Sample size	Tolerance level
100	0.63
200	0.87
300	0.95
400	0.98
500	0.99

reference population studies that included at least 300 individuals and used highly sensitive methods for cTnI or cTnT [28, 38, 45, 46]. A study from our laboratory [28] included 692 apparently healthy subjects (311 males and 381 females) with a mean (SD) age of 45.3 (17.3) years [range 11–89 years; females 46.5 (17.3) years, males 43.8 (17.1) years]. Our study found significant gender-based difference in cTnI values (men: median 0.012 μg/L, range from undetectable values to 0.196 μg/L; women: median 0.008 μg/L, range from undetectable values to 0.130 μg/L; $p < 0.0001$) using the ADVIA TnI-Ultra method (Siemens Medical Solutions Diagnostics SrL). Undetectable cTnI concentrations were found in 168 individuals (24.3% of the samples tested). All individuals used in this study were screened for preventive medicine programs (laboratory staff, blood donors, or voluntary subjects), with no acute or chronic diseases (excluded by history, accurate clinical examination, ECG, and laboratory tests), nor use of drugs for at least 2 weeks before the sample collection. A gender-dependent cut off value was also found in another study [45] that used the recently refined Access AccuTnI assay (Beckman-Coulter) to assess the distribution of cTnI in a community population of elderly individuals [PIVUS (Prospective Study of the Vasculature in Uppsala Seniors) study; $n = 1005$]. Gender-dependent differences in 99th URL for the highly sensitive cTnT assay by Roche Diagnostics was also reported by Mingels et al. [46] in a reference population of 479 apparently healthy individuals; the observed 99th percentile was 0.008 μg/L in 215 females and 0.018 μg/L in 264 males ($p < 0.001$).

In contrast, Collinson et al. [38] reported that the calculated 99th URL of cTnI concentration, measured with the ADVIA TnI-Ultra method, was very similar to that reported by the manufacturer (0.04 μg/L) and cTnI values were not age- and gender-dependent [38]. Moreover, 165 (53.4%) of the 309 individuals (127 men, 182 women; median age 53 years, range 45–80 years) enrolled were considered to have no measurable cTnI. The individuals who participated in this study were randomly selected from a population of

ostensibly healthy individuals, accurately screened to exclude any history of vascular disease, diabetes mellitus, hypertension, heavy alcohol intake, use of cardiac medication, or pathologic echocardiogram.

Some interesting observations may be derived from these studies [28, 38, 45, 46]. First, the selection of reference population may greatly influence the calculation of 99th URL. In particular, the clinical protocol used to exclude the presence of asymptomatic cardiac disease (especially in older subjects) is likely to affect the statistical analysis with respect to distribution of cTnI measured by highly sensitive methods. Second, the presence of cTnI and cTnT measurable values in apparently healthy subjects requires physiologic explanation. Increased concentrations of cTnI and cTnT have been observed in animal models of ischemia without histologic evidence of irreversible injury [49]. Moreover, apoptotic cells have been described in normal adult hearts, suggesting that myocyte replication is a significant component of normal physiology and cellular processes, even in adults [50]. This finding will be discussed in more detail in the following section. It can be suggested that the release of cTnI from cardiomyocytes of healthy adult subjects may result from a process related to the ''physiological remodeling'' of human myocardium [28, 31].

Age-dependent increases in cTnI and cTnT in apparently healthy subjects may suggest additional pathophysiological mechanisms. It is well known that the incidence of HF progressively and steeply increases after the age of 55 years (Table 4) and that this disease is the most common cause of death in elderly people [51]. Several histological changes of myocardial tissue characterized by loss of myocytes and subsequent hypertrophy of the remaining cells and calcification of several cardiac structures can be found in most individuals with aging [52, 53]. Moreover, the age-related loss of arterial compliance contributes to isolated systolic hypertension and left ventricular hypertrophy [52, 53]. Despite these changes, for the majority of apparently healthy older

TABLE 4
PREVALENCE OF SYSTOLIC AND DIASTOLIC DYSFUNCTION BY AGE (ACCORDING TO REFERENCE 51)

Dysfunction	45–54 years	55–64 years	65–74 years	75 and older	Overall
Diastolic					
Moderate	1.4%	6.0%	9.9%	14.6%	6.6%
Severe (restrictive)	0%	0.4%	0.7%	3.4%	0.7%
Systolic					
LVEF $\leq$ 50%	3.0%	4.8%	7.1%	12.9%	6.0%
LVEF $\leq$ 40%	0.8%	1.3%	2.7%	4.4%	2.0%

LVEF, Left ventricular ejection fraction.

adults, cardiac output is well preserved by means of the Frank–Starling principle, in the setting of reduced early diastolic filling [53]. In accordance with these findings [52, 53], we suggest that increased levels of cTnI, measured with high-sensitivity immunoassay methods in some apparently healthy older adults [28, 45, 50], are likely to be due to increased remodeling of myocardial tissue in this population. This hypothesis is well in agreement with the results reported by Eggers et al. [54]. This study investigated the prevalence of cTnI elevation in an elderly community population that included 1005 individuals aged 70 years. Using a highly sensitive immunoassay, this study found that increased cTnI was relatively common in elderly subjects and was associated with cardiovascular high-risk features and/or impaired cardiac performance [54].

Cumulatively, these data strongly indicate that calculation of 99th URL is dependent on demographic and clinical characteristics of the reference population used in the study. Hence, clinical cutoffs using highly sensitive cTnI and cTnT assays should be based on analytic definitions (i.e., CV) versus distribution characteristics (i.e., percentiles) such that "true" troponin increase may be identified [38]. Furthermore, the demographic and clinical characteristics of the reference population enrolled for calculation of 99th URL should be clearly delineated by the commercial manufacturers as well as authors of published clinical studies.

5.2. More Myocardial Infarctions or More False Positive Results?

It is reasonable that the new generation of high sensitivity cTnI and cTnT methods can detect a greater number of patients with AMI than standard methods, especially those individuals with very small infarct size [5, 55]. Data reported by some recent studies appear to confirm this hypothesis [19, 55–59]. Unlike most commercial cTnI and cTnT methods, the use of highly sensitive methods (i.e., with increased assay sensitivity at very low troponin concentration) will allow earlier diagnosis of AMI (within 1–2 h after thoracic pain onset), as well as recognition of very small (focal) areas of myocardial necrosis as true AMI.

Although professional societies have recognized the importance of the enhanced analytic performance of the newer and emerging cardiac troponin assays, the clinical community has not uniformly embraced this trend [55]. Indeed, it is uncertain if the new highly sensitive troponin assays will lead to increased clarity or more confusion for most physicians [59]. Predictably, the application of assays with lower limits of detection has led to an increase in patients evaluated in the emergency setting with detectable cardiac troponin in a variety of acute and chronic medical conditions other than ACS [5, 16, 19, 31, 55, 56].

It is also reasonable that increased cardiac troponin in apparently stable populations, such as elderly subjects from the community and patients with previous ACS, may primarily reflect left ventricular hypertrophy and/or myocardial pump failure with a continuous loss of viable cardiac myocytes caused by increased myocardial wall strain, chronic ischemia, or apoptosis. It is well known that these conditions are often associated with ST-T segment abnormalities that may mimic changes related to acute coronary ischemia. Accordingly, several studies have already demonstrated that more sensitive troponin assays increased the number and rapidity of AMI diagnosis, but also increased the number of false positives, that is, non-ACS-related pathology [16, 19, 25, 56, 57]. In fact, Eggers et al. [16] reported a $\sim 7\%$ misdiagnosis (i.e., false positivity rate) for AMI in troponin-positive patients with preexisting ST-T segment abnormalities in patients admitted for nonischemic chest pain or other symptoms indicative of myocardial ischemia.

In conclusion, these data confirm that it is very difficult (or even impossible) to reliably diagnose patients suspected with ACS using only one determination of cTnI or cTnT due to the relatively low specificity of existing cardiac troponin assays for ischemic myocardial injury. Indeed, international guidelines [1, 2] recommend at least two samples with a delay of time of 6–12 h for measurement of cTnI and cTnT in these settings.

5.3. Clinical Relevance of Serially Measured Troponin Circulating Levels

It is important to note that the detection of a true and significant increase/decrease in serially measured troponin is of critical importance to correctly establish the diagnosis of AMI in all patients without a diagnostically reliable or recent electrocardiogram [1, 2] (Fig. 2) and to discriminate between ischemic and other causes of troponin increase [1, 2, 16, 35, 60, 61].

Unfortunately, there is no consensus about the required degree of change for serial measurement of cTnI and cTnT in AMI diagnosis in patients with suspected ACS. The National Academy of Clinical Biochemistry has recommended a 20% change as statistically significant [60]. However, these recommendations assume that analytical assays have a precision of 5–7% with three times the SD and produce 99% confidence at limit at the AMI decision point [61].

A statistically more rigorous approach toward assessment of meaningful serial markers in clinical laboratory tests would be to first ascertain biologic variation [14, 35]. Unfortunately, troponin biologic variation cannot be evaluated with certainty using the standard methods due to their inability to detect the protein in the blood of healthy subjects with adequate precision. Using new high-sensitivity assays, Wu et al. [35] were able to demonstrate

that cTnI biological variation was lower than other cardiovascular biomarkers, that is, cardiac natriuretic peptide hormone, creatine kinase-MB fraction (CK-MB), myoglobin, C-reactive protein (CRP), myeloperoxidase, and serum amyloid A [35]. Because this study was performed in healthy subjects, there is concern regarding the applicability and reliability of these biologic variation parameters in patients with ACS. One would expect that there are differences in biologic variation parameters between healthy individuals and patients with ACS. In comparison to cTnI, cTnT has different release kinetics from myocytes and clearance in peripheral tissues and therefore its biologic variation should be evaluated in specific studies [35]. Clearly, more comprehensive studies are required to confirm that measurement relative to biologic variation is useful in evaluating the clinical significance of cardiac troponin in patients with ACS.

5.4. High-Sensitivity Troponin Methods in Patients with HF: A Better Stratification of Cardiovascular Risk

HF is a major public health problem in the North America and Europe [62–65]. The incidence and prevalence of HF increases significantly with aging in these populations. After the age of 65, the incidence of HF approaches 10 per 1000 of population ($\sim$1:100) [51]. In the United States, HF is the most common hospital discharge diagnosis, and more Medicare dollars are spent for diagnosis and treatment of HF than for any other disease [62–64]. Similar data have been reported from European countries [65].

HF may be considered as the fatal progression of all cardiovascular disorders. For this reason, HF is considered a syndrome rather than a primary diagnosis which results from any structural or functional cardiac disorder that impairs the ability of the heart to function as a pump to supporting physiologic circulation [5, 31]. We can assume that, if heart dysfunction is an inevitable and ultimate fate, the measurement of some highly specific cardiac biomarkers, such as cTnI, cTnT, B-type natriuretic peptide (BNP) and its related forms, should be useful in detection of people at risk of a more rapid progression toward symptomatic HF, thus in need of specific clinical treatment [5, 31].

In 2001, the AHA/ACC task force for the diagnosis and management of chronic HF introduced a new classification that focused on disease evolution [62]. This classification, updated in 2005 [63] and 2009 [64], identified four stages (A, B, C, and D) that account for symptoms, established risk factors for HF development and structural myocardial abnormalities. Stage A includes asymptomatic patients at risk for developing HF with no structural cardiac involvement. Stage B includes asymptomatic patients at risk for developing HF with structural cardiac involvement. Stage C includes

patients with past or current symptoms of HF associated with underlying structural heart disease. Stage D includes symptomatic patients with end-stage disease who require specialized treatment strategies, such as mechanical circulatory support, continuous inotropic infusions, cardiac transplantation, or hospice care. Unlike the NYHA classification which is based on clinical severity of symptoms, this new classification emphasizes the progressive nature of the pathophysiological processes responsible for development of HF [64]. In fact, the first two stages (A and B) clearly do not include HF but help in the early identification of patients at risk for developing HF [64]. Stages A and B patients are best defined as those individuals with risk factors that clearly predispose to the development of HF.

Appropriate risk stratification depends on the availability of specific, accurate, and effective disease and risk markers [5, 31]. Highly sensitive cTnI and cTnT immunoassay methods share the most important analytic and clinical performance characteristics of an ideal cardiac biomarker (Table 5) [5, 31].

It is well known that a relatively large proportion of HF patients (25–45%), especially those with clinical history of coronary artery disease, has increased cTnI and cTnT, even if measured by standard (i.e., not highly sensitive) methods [64]. Recent studies [16, 54, 58, 59] have suggested that the fraction of patients with HF and troponin values above the 99th URL may further increase when highly sensitive cTnI and cTnT assays are used. In particular, the ValHeFT study (including 4053 randomized patients with symptomatic heart failure) demonstrated cTnT values above the cut-off level in 10.4% of patients studied using a standard assay and this percentage increased to 92% when a more sensitive method was used [66].

TABLE 5
DESIRABLE FEATURES OF AN IDEAL MOLECULAR CARDIAC BIOMARKER

- Absolute cardiospecificity
- Acceptable to patient
- Stability *in vivo* and *in vitro*
- Adequate analytical sensitivity (functional sensitivity) easy to perform
- Good degree in reproducibility and accuracy
- Complete automation of assay
- Internationally standardized
- Low cost
- Low biological variation
- Reference range and cut off values tested for gender, age, and ethnicity dependence
- Good diagnostic and prognostic accuracy
- Favorable cost-benefit ratio

It is well known that troponin and natriuretic peptide, when used as biomarkers of cardiac disease, furnish complementary clinical information [5, 31]. The progressive increase in both markers with aging [5, 20, 28, 31, 45] suggests that there is progressive decline of cardiac function which can be assessed and monitored. If heart dysfunction is an inevitable and ultimate fate, the measurement of these analytes would be useful to initiate specific clinical care for those individuals at risk of more rapid progression to symptomatic cardiac failure.

5.5. Early Detection of Myocardial Injury in Patients with Extracardiac Diseases or Assuming Potentially Cardiotoxic Drugs

The Dallas Heart Study (3357 subjects) found that the prevalence of increased cTnT in the general population was $\sim 1\%$ using electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics), a standard assay [67]. The prevalence of high-risk cardiovascular features was increased similarly in subjects with cTnT levels in the minimally increased and increased range [67]. These data suggest that cTnT elevation is always indicative of cardiovascular disease or at least a high-risk cardiovascular profile. As such, increased cardiac troponin represents an important finding even in those patients without coronary artery disease. This ''index'' of cardiac tissue damage may suggest an appropriate diagnosis and, when necessary, a specific treatment. Increased cTnI or cTnT in patients without evidence of ischemic coronary artery disease represent an independent risk of future cardiac events and poor prognosis [5, 31, 60]. It is important to point out that there is no minimal threshold and that cardiovascular risk increases progressively with increased troponin.

Increased troponin has been reported in patients treated with potentially cardiotoxic drugs, such as high-dose chemotherapy [68, 69]. Indeed, a specific pattern of troponin release following high-dose chemotherapy identified at-risk patients for cardiac events in the subsequent 3 years [68]. Furthermore, in patients treated with high-dose chemotherapy, increased cTnI suggested early and appropriate treatment and was found to prevent the development of late cardiotoxicity [69].

Another important application of highly sensitive troponin assay (along with cardiac natriuretic peptides) may be the early detection of myocardial damage in patients with systemic acute or chronic inflammatory and rheumatic diseases (i.e., systemic lupus erythematosus, systemic amyloidosis, sarcoidosis, and rheumatoid arthritis) [70–73]. It is noteworthy that mortality risk in these patients is strongly associated with heart complications versus other organ involvement [72, 74–76]. As a result, the early detection of cardiac involvement may have a tremendous clinical impact on the prognosis

of patients with chronic inflammatory and rheumatic diseases, especially those with systemic amyloidosis [72]. Early identification of these patients is critical in the initiation of successful treatment strategies.

It is well known that cardiovascular events are also the major prognostic determinants in patients with end-stage renal disease, with cardiovascular deaths representing more than 50% of total mortality [77, 78]. The early recognition of conditions such as left ventricular hypertrophy and coronary artery disease may allow the identification of patients with chronic kidney disease at higher risk of developing either HF or other major cardiovascular events with consequent increased mortality [77, 78]. In a considerable number of chronic hemodialysis patients, increased troponin was found despite absence of cardiac ischemia, even if older generation cTnI and cTnT immunoassay methods were used [77, 78]. The elevation of cardiac biomarkers in patients with renal diseases showed a strong prognostic significance with respect to cardiovascular morbidity and mortality [5, 31, 73, 77, 78]. Recent studies have shown that highly sensitive cTnI and cTnT immunoassays were able to detect a greater number of end-stage renal disease patients with increased troponin [58, 78, 79]. As such, high-sensitivity cTnI and cTnT immunoassays may provide a useful rationale for both occult cardiac disease screening and better cardiovascular risk stratification in this unique group of patients.

In conclusion, sensitive cTnI and cTnT immunoassays may provide a useful tool for the early screening of occult cardiac disease either in patients with extracardiac diseases (especially renal and chronic inflammatory diseases) or in subjects undergoing treatment with potentially cardiotoxic drugs (such as high-dose chemotherapy). However, further studies are necessary to demonstrate the clinical impact and the cost-effectiveness of this approach according to the evidence-based laboratory medicine principles (EBLM) [80].

6. High-Sensitivity cTnI and cTnT Methods: A Powerful Tool for Monitoring Physiological Renewal and Pathological Remodeling of the Myocardial Tissue?

Increased analytical sensitivity of cTnI and cTnT methods has demonstrated that measurable levels of these proteins are also present in apparently healthy subjects [20, 28, 38] (Fig. 3). These findings suggest some interesting pathophysiological considerations. At present time, the prevailing opinion, based on the aggregate evidence to date, is that any reliably detected elevation of a cardiac troponin is abnormal and might represent cardiac necrosis [81]. However, apoptotic cells have been described in normal adult hearts; thus suggesting that myocyte replication is a significant component of the

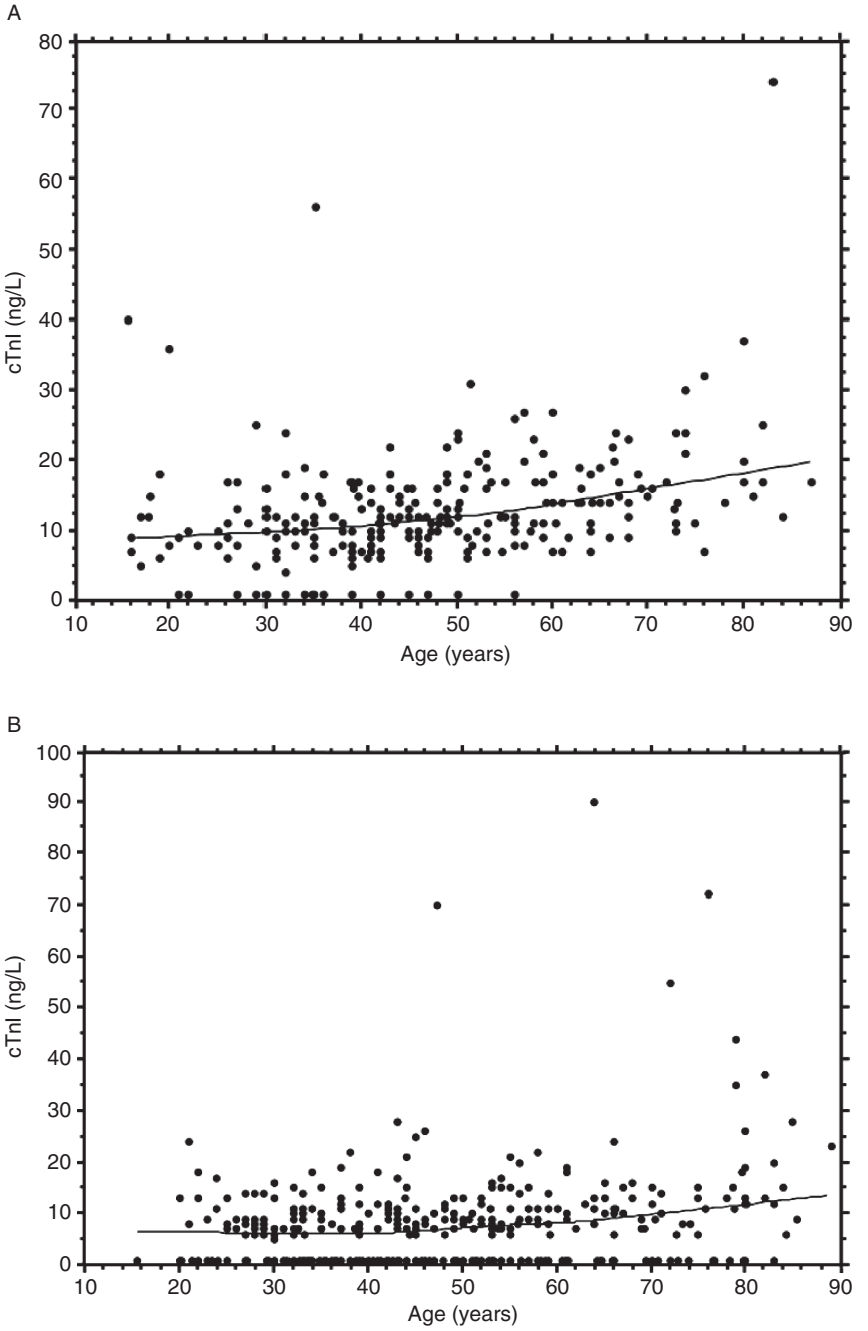

FIG. 3. (Continued)

physiological cellular processes even in adults [50]. A very recent experimental study, based on DNA integration of the isotope ^{14}C generated by nuclear bomb tests during the Cold War, was able to establish cardiomyocyte age in humans [82]. The results of this study suggested that cardiomyocytes renew with a gradual decrease of annual turnover from 1% (age 25) to 0.45% (age 75) with fewer than 50% of cardiomyocytes exchanged during a normal life span [82].

At present time, there are no experimental data indicating that troponins are degraded within the cardiomyocytes and released into the interstitial space during apoptosis. There are two potential explanations for the troponin release in absence of lethal sarcolemmal disruption: (1) cellular release of proteolytic troponin degradation products; (2) troponin leaks as an intact nondegraded protein chain from reversibly damaged cardiomyocytes [83, 84]. Mechanical stretch of cardiomyocytes, for example, during pressure or volume overload, may activate some intracellular proteases, such as metalloproteinase, that can degrade cardiac troponin intracellularly [85]. Overload-induced stretch at the cardiomyocyte level is sensed by integrins, which are mechanotransducer molecules that link the extracellular matrix to the intracellular cytoskeleton [86]. Hence, this mechanism may be involved in stretch-induced release of troponin and its degradation products [83]. These findings suggest that stretch stimulation of viable cardiomyocytes may lead to intact cTnI release. Indeed, several studies have demonstrated that mechanically induced transient disruptions (wounding) of the sarcolemma are a constitutive *in vivo* event [87–90]. This mechanism may account for the release of proteins, like myocyte-derived growth factors that are released despite lack of the classic signal peptide sequence that is normally associated with secretion. These mechanically induced alterations in cardiomyocyte sarcolemmal permeability may similarly be involved in the release of cTnI from cytosolic pools in the absence of necrotic cell death. However, further studies are necessary to accurately describe the cellular mechanisms responsible for release of intact cTnI and cTnT in damaged cardiomyocytes.

FIG. 3. (A) Age-dependent distribution of cTnI values measured by the ADVIA method on the Centaur Platform (Siemens Diagnostics) in 269 apparently healthy male subjects (age ranging from 14 to 88 years). There is a very weak, although significant, correlation between age and cTnI values (by Spearman Rank test, Rho $= 0.358$, $p < 0.0001$). The trend, assessed by smooth spline analysis, between age and cTnI values is indicated by a continuous line. (B) Age-dependent distribution of cTnI values measured by the ADVIA method on the Centaur Platform (Siemens Diagnostics) in 238 apparently healthy female subjects (age ranging from 14 to 88 years). There is a very weak, although significant, correlation between age and cTnI values (by Spearman Rank test, Rho $= 0.258$, $p < 0.0001$). The trend, assessed by smooth spline analysis, between age and cTnI values is indicated by a continuous line. Results obtained in the Authors' laboratory (see references 21, 28, 29).

The data, regarding gender- and age-related cTnI and cTnT levels in adult healthy subjects [21, 23, 28, 45], support the hypothesis that small amounts of cTnI can be released from cardiomyocytes even in apparently healthy subjects due to a process related to the "physiological renewal or remodeling" of human myocardium. Moreover, some findings obtained in healthy individuals after endurance exercise appear to confirm this hypothesis. Several studies reported increased circulating cTnI or cTnT after strenuous exercise (such as marathon runs or other endurance races), even in well-trained athletes [91–98]. Middleton et al. [95] suggested that it is unlikely that minor elevations in cTnI or cTnT subsequent to endurance exercise are due to myocardial necrosis. These authors hypothesized that postexercise troponin release represents the reversible cardiomyocyte membrane damage during remodeling processes [95]. According to this hypothesis, in a healthy exercising population, cardiac troponins may be routinely released after periods of increased myocardial demand, such as after endurance exercise. As mentioned earlier, recently developed methods for cTnI and cTnT assays may be able to detect a release of protein from a quantity of myocardial tissue of a few milligrams in size [20–30] (Table 1).

Highly sensitive cTnI and cTnT immunoassays should be considered a useful and potentially powerful tool to monitor the continuous and physiological processes related to renewal and remodeling of the myocardial tissue.

7. Use of High-Sensitivity cTnI and cTnT Methods in a Multimarker Approach for Early Screening: An Increase in Diagnostic and Prognostic Efficiency?

Many biomarkers exist for the diagnosis and prognosis of individuals with cardiovascular diseases. Methods of assessment using a multimarker approach have been considered the best model for risk prediction in individuals with cardiovascular disease [5, 31, 99]. Despite the many suggested laboratory biomarkers for diagnosis, risk assessment, and follow-up of patients with cardiovascular disease, only cardiac troponins and natriuretic peptides have been shown to have good diagnostic and prognostic accuracy, as well as cost-effectiveness, according to EBLM principles [5, 31, 99].

Indeed, these two biomarkers are characterized by high analytical and clinical sensitivity as well as by absolute cardiospecificity. Cardiac troponins and natriuretic peptides have different physiological roles. Troponins are structural proteins of actin–myosin complex involved in cardiac contraction and relaxation, whereas the latter are peptide hormones with natriuretic and vasodilative effects produced by cardiomyocytes. Furthermore, different pathophysiological mechanisms affect the release of cardiac troponins and

natriuretic peptides by cardiomyocytes [5, 31, 100–103]. Owing to their different pathophysiological roles, cardiac troponins and natriuretic peptides may provide independent pathophysiological and clinical information. Due to their unique roles, these markers may be combined in a multimarker model for cardiovascular risk assessment and as predictive variables in practically every acute and chronic cardiac disease, including those due to ischemic, inflammatory, congenital, and traumatic conditions. A substantial number of studies have confirmed this hypothesis [5, 13, 31, 60, 81, 104–109]. Furthermore, both biomarkers can be measured using highly sensitive immunoassays with detection limits below 10 pg/mL [5, 31, 108]. As such, these biomarkers may be useful to assess disease progression and severity in patients with stage B HF (ACC/AHA guidelines) [62–64] and patients with end-stage renal disease [76]. Although some recent studies provide some support [25, 54, 109–111], further and more comprehensive studies are required to demonstrate the effectiveness of highly sensitive cTnI and cTnT, alone or together with BNP, in early screening of cardiac disease in the general population or in high-risk individuals.

8. Conclusion

Improvement of analytical precision and clinical sensitivity of a laboratory test is a goal for all laboratorians. This concept should be applied to cTnI and cTnT immunoassays.

Careful evaluation with development of new diagnostic criteria (including 99th URL and decisional cut-off values) will be necessary to improve patient care. More accurate and earlier diagnosis should lead to more effective therapies. In particular, highly sensitive cTnI and cTnT assays will better define the true normal levels and the 99th URL. Moreover, the new generation of troponin assays will enable better risk stratification of patients who do not have increased troponin by current assays and may allow risk stratification of patients with chronic stable angina and patients with HF. It is really important to stress that increased cTnI and cTnT represent an index of cardiac tissue damage, even in the case of extracardiac diseases (including chronic inflammatory disease, end-stage renal disease, or treatment with powerful cardiotoxic drugs), suggesting an appropriate diagnosis and, when necessary, a specific treatment. Despite these interesting and preliminary studies, further and more comprehensive studies with highly specific assays are required to firmly establish the clinical usefulness of troponin in a wide range of diseases.

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