

## Review

Aldo Clerico\*, Martina Zaninotto, Andrea Ripoli, Silvia Masotti, Concetta Prontera, Claudio Passino and Mario Plebani, on the behalf of the Study Group on Cardiovascular Risk Biomarkers of the Italian Society of Clinical Biochemistry (SIBioC)

# The 99th percentile of reference population for cTnI and cTnT assay: methodology, pathophysiology and clinical implications

DOI 10.1515/cclm-2016-0933

Received October 14, 2016; accepted February 28, 2017; previously published online April 12, 2017

**Abstract:** According to recent international guidelines, including the 2012 Third Universal Definition of Myocardial Infarction by the Joint ESC/ACCF/AHA/WHF Task Force, an increase in cardiac troponin (cTn) levels over the 99th percentile upper reference limit (99th URL) should be considered clinically relevant, this cut-off being measured with an imprecision  $\leq 10$  CV%. In theory 99th URL values strongly depend not only on demographic and physiological variables (i.e. criteria for considering the reference population “healthy”), but also on the analytical performance of cTn methods and mathematical algorithms used for the calculation. The aim of the present article was therefore to review the methodological and pathophysiological factors affecting the evaluation and calculation of the 99th URL for cTn assay. The critical analysis made showed that no uniform procedure is followed, and nor have experts or regulatory bodies provided uniform guidelines for researchers or cTn assays manufacturers as an aid in “their quest to define normality”. In particular, little attention has been paid to the way in which a healthy reference population is to be selected, or the criteria for calculating the 99th URL value for cTn assays, thus highlighting the need for international recommendations not only for demographic and physiological variables criteria

for defining a healthy reference population, but also for calculating mathematical algorithms for establishing/calculating clinical decision values. An expert consensus group, comprising laboratory and clinical scientists, biomedical statisticians, industrial and regulatory representatives, should be responsible for drawing up these guidelines.

**Keywords:** cardiac troponins; gender specific decision values; highly sensitive immunoassay; quality specification; reference population.

## Introduction

The international guidelines “on the Redefinition of AMI”, issued in the year 2000 by the Joint European Society of Cardiology/American College of Cardiology Committee, recommended that an increase in cardiac troponin I (cTnI) or cTnT levels outside the 99th percentile upper reference limit (99th URL) should be considered clinically relevant, and indicated that this cut off value should be measured with an imprecision of  $\leq 10$  CV% [1]. Subsequently the guidelines for the Universal Definition of Myocardial Infarction, edited by the joint ESC/ACCF/AHA/WHF task force in 2007 [2] and 2012 [3], confirmed that cTnI and cTnT are the preferred biomarkers for the differential diagnosis of acute coronary syndrome (ACS), and also that the 99th URL value should be measured with an imprecision of  $\leq 10$  CV%.

Quality specifications for troponin assay require the presence of measurable cTnI and cTnT, also in the blood of healthy subjects [4–6]. However, measurement of the 99th URL of cTnI and cTnT levels is a challenging analytical issue due to low biomarker concentrations present in healthy subjects [7–9]. Only after 2006 some manufacturers set-up the first new generation of cTnI and cTnT immunoassays with improved analytical sensitivity in accordance

**\*Corresponding author: Professor Aldo Clerico, MD,** Laboratory of Cardiovascular Endocrinology and Cell Biology, Department of Laboratory Medicine, Fondazione CNR Toscana G. Monasterio, Scuola Superiore Sant’Anna, Via Trieste 41, 56126 Pisa, Italy, E-mail: clerico@ftgm.itr

**Martina Zaninotto and Mario Plebani:** Department of Laboratory Medicine, University-Hospital, Padova, Italy. <http://orcid.org/0000-0002-0270-1711> (M. Plebani)

**Andrea Ripoli, Silvia Masotti, Concetta Prontera and Claudio Passino:** Fondazione CNR Regione Toscana G. Monasterio and Scuola Superiore Sant’Anna, Pisa, Italy

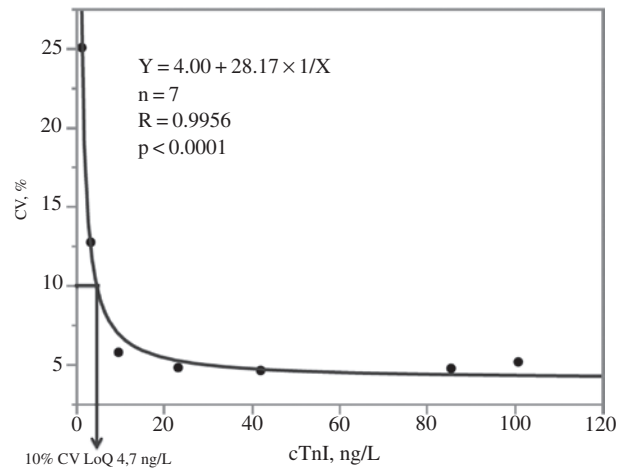
with the quality specifications indicated in international guidelines and consensus documents [4, 6–16]. Importantly, highly sensitive methods should also be able to measure troponin levels in the majority of healthy adults subjects (>50%), as suggested in some consensus documents [16–18]. In order to measure the extremely low circulating levels of the biomarker in all healthy subjects, a highly sensitive method for cTnI or cTnT should have a low detection limit greatly lower than 5 ng/L [8].

Currently few methods able to completely meet the quality specifications recommended by international guidelines are commercially available for cTn assay [9, 16–18]. In one case, for example the manufacturer reports that the limit of blank (LoB) and limit of detection (LoD) for the highly sensitive cTnT method are 2.16 ng/L and 4.72 ng/L, respectively, using the Elecsys 2010 and the Cobas e411 analyzers and 1.36 ng/L and 2.05 ng/L, respectively, using the MODULAR ANALYTICS E170, Cobas e601 and Cobas e602 platforms, respectively (package insert of Troponin T hs REF 05092744 190, 2016-06, V7.0, Roche Diagnostics GmbH, Mannheim, Germany). On the other hand, the “STAT Architect highly sensitive TnI” method shows LoB, LoD and LoQ (quantitation limit at 10% CV) values of about 0.7 ng/L, 1.3 ng/L, 5.0 ng/L, respectively, as reported by the manufacturer (package insert of STAT Highly sensitive Troponin I, REF 3P25, B3P250, G1-0139/R02), and in two studies in the literature (Table 1) [14, 15].

The most important analytical characteristic for a highly sensitive troponin assay, is the ratio between the 99th URL and LoQ values at 10% CV, which should be greater than 1 [5]. Considering that the 99th URL value for the cTnI Architect method recommended by manufacturer is 26.2 ng/L, this value is 4-fold higher than the LoQ at 10% CV. Furthermore, the 99th URL value (i.e. 26.2 ng/L) is measured by the Architect method with an imprecision of about 5% CV (Figure 1), which is half the imprecision required by international guidelines for the 99th URL value (i.e. 10% CV) [NaN]. The Architect method can also detect cTnI levels that exceed LoD in more than 85% of healthy subjects including children and adolescents [14, 15], as requested by some consensus documents [16–18]. These data, shown in Table 1 and

**Table 1:** LoB, LoD and LoQ values of hs-STAT Architect immunoassay for cTnI.

Reference	LoB, ng/L	LoD, ng/L	LoQ CV 10%, ng/L
Manufacturer	0.7–1.3	1.1–1.9	4.7
Krintus et al. [14]	0.7–1.3	1.1–1.9	4.6–8.1
Caselli et al. [15]	0.7	1.3	4.7



**Figure 1:** Imprecision profile of the Architect method for cTnI assay. The imprecision profile was obtained in the Authors' laboratory by measuring in 39 different runs seven plasma pools collected from healthy subjects and patients with cardiac disease using three different lots of reagents and calibrators throughout 2 months.

Figure 1, clearly indicate that the Architect method has the analytical performance required for a true highly sensitive method for cTnI assay.

There are further methodological considerations to take into account in explaining the great differences between the analytical performances between the cTn assay methods reported in the literature. There is no general consensus on the evaluation and calculation of the imprecision profile and, consequently, of the LoQ value [19]. Manufacturers and authors often fail to report in detail how the imprecision profile and LoQ at 10% CV values are calculated. Even international CLSI guidelines [20, 21] for the evaluation of the imprecision profile have some limitations, especially when between-runs variability, requiring multiple recalibrations, is recommended [19]. The seminal studies by Spencer et al. [22] on TSH immunoassays clearly demonstrate that the imprecision profile of immunometric assays strongly depends on the number of reagents lots and instrument calibrations involved; these data suggest that lot-to-lot variation of material and calibrators have an impact on the calculation of 99th URL values. Spencer et al. [22] also recommend that between-runs ( $n > 10$  runs) imprecision should be evaluated across 6–8 weeks, using more than one batch of reagent, and more than one calibration instrument.

Furthermore, LoQ values (10% or 20% CV) can vary significantly when different functions are used for fitting the relationship between response error (i.e. CV%) and the level of response (i.e. analyte concentration), such as straight line, parabolic functions, power equations or variance models, using three or four parameters [19]. For

example the data reported in Figure 1 give a value of 4.7 ng/L for LoQ at 10% if fitted by a reciprocal function (as shown), but a value of 6.5 ng/L, if the same data are fitted by a power function (i.e.  $Y = 19.764 * X^{-0.359}$ ), which actually shows a worse fit.

The study by the Committee on Standardization of Markers of Cardiac Damage of the IFCC, published in 2004, found a difference of up to 20-fold between the 99th URL values of cTnI and cTnT immunoassays [5]. Despite the great improvement achieved in the analytical performance of cTnI immunoassays over the last few years [8], two recent studies demonstrated the persistence of great systematic differences between the results of commercial cTnI methods (on average up to more than 2-fold) [9, 23]. The lack of harmonization among the troponin immunoassay methods may generate confusion in physicians, with a consequent misinterpretation of cTnI and cTnT results [8, 23, 24].

The aim of the present article was therefore to review and make a critical analysis of the methodological issues and pathophysiological mechanisms affecting the evaluation and calculation of 99th URL for cTn assay.

## Criteria for calculating 99th URL values

It is conceivable that 99th URL values not only strongly depend on demographic and physiological variables (i.e. the criteria for considering the reference population “healthy”) [17, 25–29], but also on the analytical performances of cTn methods, as well as on the mathematical algorithms used for calculating the 99th URL [10, 14, 15, 17, 18, 25, 26].

## Criteria for selecting reference population

The approach for selecting a reference population for calculating the 99th percentile value for cTn assays has not yet been adequately defined [17]. The Third Universal Definition of Myocardial Infarction [3] reported that gender-dependent values might be recommended for highly sensitive troponin assays without specifying criteria for considering a population “healthy”. The ESC guidelines issued in 2010 [30] state that the calculation of the 99th percentile is markedly affected by outliers; consequently, to reach a 95% confidence value in the 99th URL calculation, a sample size of at least 300 individuals per group is required [31]. They also state that

meeting these requirements is challenging and costly, and most laboratories have no resources to perform studies to identify troponin reference limits [30]. The ESC guidelines [32], issued in 2016 for the management of ACS in patients without persistent ST-segment elevation, fail to address this issue [32].

## Age

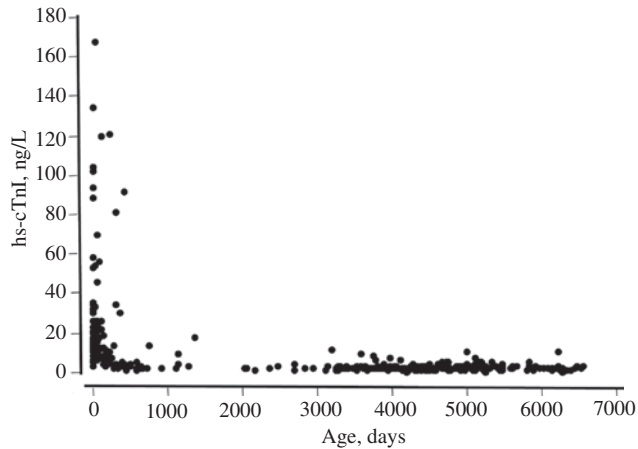
Several studies appearing since 2005 have used the first generation of immunoassay methods with increased analytical sensitivity for cTnI and cTnT assay, and have demonstrated that measurable troponin values are present in blood of the majority of healthy adults [7, 8]. Thanks to the ensuing improvement achieved in the analytical sensitivity of immunoassay methods, it is now possible to detect cTnI and cTnT levels throughout the lifespan, from birth to senescence [9–15, 17, 18, 33, 34].

Regarding the pediatric age, some studies issued since 2012 using the highly sensitive method with Architect platform, demonstrated that cTnI levels (i.e. concentration values higher than the LoD value of 1.3 ng/L) were clearly evidenced in more than 85% of a population of apparently healthy children (ages ranging from birth to adulthood) when a highly sensitive method was used for troponin measurement [15, 33, 34]. cTnI levels, at their highest in the first few weeks of life, tended to gradually decrease up to adulthood [15] (Table 2 and Figure 2). From a physiological viewpoint, these data suggest that cTnI is released from cardiomyocytes throughout the pediatric age, probably in relation to physiological growth. However, further studies are needed to confirm these preliminary data, and to establish cTnI reference intervals from neonatal age to adolescence. As yet no reference interval data are available for cTnT in the pediatric age, circulating levels being mainly below the LoD value of ECLIA method for cTnT assay [26].

Several studies published in the time period between 2005 and 2010 report that cTnI levels progressively

**Table 2:** cTnI values of neonates, children and adolescent subjects according to age measured with the high sensitive method using the ARCHITECT platform, according to reference [15].

Age	Number	Median, ng/L	Range, ng/L
1 ≤ days < 30	36	21.5	12.9–44.0
1 < months ≤ 12	57	11.5	5.7–21.7
1 < years ≤ 10	65	2.2	1.6–2.8
10 < years ≤ 18	221	2.0	1.5–2.8
0 < years ≤ 18	379	2.5	1.7–6.2



**Figure 2:** cTnI concentrations measured with highly sensitive ARCHITECT assay in apparently healthy neonates, children and adolescents.

increase in apparently healthy adult subjects aged over 65 years [10, 13]. This finding was confirmed in studies using the highly sensitive cTnI method [14, 27]. The progressive increase of circulating cTnT levels in apparently healthy adult subjects over 65 years was confirmed in the same period, when the highly sensitive ECLIA method became commercially available [11, 12, 26, 35–37]. In their study on a total of 651 subjects admitted to emergency department (ED) without evident conditions known to increase cTnT, Hammarsten et al. [36] found cTnT values over the cut-off (i.e. 14 ng/L) in 9% of subjects admitted to ED, most of whom (>90%) were  $\geq 65$ -year olds. Furthermore, in subjects aged <65 years admitted to ED without evident conditions known to cause an increase in cTnT, the 99th cTnT percentile was 12 ng/L, with little age dependence whereas the 99th cTnT percentile, at 82 ng/L, was highly age dependent in subjects aged  $\geq 65$  years [36].

The ESC international guidelines on the use of high-sensitivity cTn in acute cardiac care, published in 2010 [30], indicated that the reference population should ideally have negative exercise stress tests and normal cardiac function assessed by imaging without providing further specifications regarding the age range of subjects enrolled. Nor do the 2012 ESC guidelines [38] or the Third Universal Definition of Myocardial Infarction [3] take into account the issue of the definition of the reference population for calculating the 99th URL.

Due to the difficulty involved in enrolling truly healthy subjects aged  $\geq 65$  years [17, 26], the 99th URL values for cTnI and cTnT reported in clinical studies or by manufacturers are commonly calculated in populations aged between 20 and 65 years, usually recruited from blood donors. For example, in the instruction

manual, the manufacturer of the highly sensitive ECLIA method reports that the 99th URL value of 14 ng/L (limit of confidence at 95%: 12.7–24.9 ng/L) was calculated in a population of 533 healthy volunteers without specifying gender and age range (instruction manual: Troponin T hs, REF 05092744 190, 2011-02, V4, Roche Diagnostics). Yet in the instruction manual of the ARCHITECT STAT Highly sensitive Troponin-I (package insert of STAT Highly sensitive Troponin I, REF 3P25, B3P250, G1-0139/R02, Abbott Diagnostics), the manufacturer indicates two 99th URL values differentiated on the basis of gender, reporting that the reference population comprised 765 females (age range, 21–75 years; 99th URL 15.6 ng/L) and 766 males (age range, 21–73 years; 99th URL 34.2 ng/L), respectively.

In view of the above, the 99th URL values of highly sensitive methods for cTnI and cTnT currently used in clinical practice are underestimated for age [35, 39, 40]. Data reported by Franzini et al. [26], for example, show that the calculated 99th URL values for cTnT ECLIA method in apparently healthy elderly men and women (>65 years) were 36.8 ng/L and 28.6 ng/L, respectively, whereas the 99th URL value advocated by the manufacturer is 14 ng/L. Furthermore, Gore et al. [41] reported that the use of a uniform 14 ng/L cutoff for the hs-cTnT assay may lead to over-diagnosis of AMI, particularly in men and the elderly.

In theory, the use of underestimated 99th URL values in clinical routine should increase clinical sensitivity, but decrease troponin assay specificity [42]. This issue is of great clinical relevance in view of the fact in ED patients an enormous number of cardiac and even extra-cardiac clinical conditions are associated with cTnI or cTnT levels higher than 99th URL [1–3, 38]. Indeed, according to Giannitsis and Katus [40], only about 40% of patients admitted to ED with at least one troponin value over the 99th URL measured by highly sensitive methods actually has AMI.

Higher circulating levels of cTnI and cTnT in elderly healthy subjects might be caused by an increased release of these proteins from cardiomyocytes, by a decreased turnover (e.g. due to reduced glomerular filtration rate), or by the combined action of these two mechanisms. Indeed, as recently shown in detail [43, 44], several age-associated disorders can cause the death of cardiomyocytes, with the consequent release of sarcolemma proteins, including cTnI and cTnT. On the other hand, circulating cTnI and cTnT are fragmented into molecules small enough to be cleared by the kidney of healthy subjects [45]. However, there is little evidence that decreased renal clearance can increase cTn levels [46].

## Gender

Only after 2007, thanks to the availability of the first highly sensitive cTnI and cTnT methods, it was possible to demonstrate that circulating troponin values are gender-dependent [10]. In a study published in 2008 [7] on 692 apparently healthy subjects (311 M, 381 F; mean age 45.3 [range 11–89] years), a significant difference was found between men and women for cTnI values (M, median 12 [range 7–96] ng/L; F, median 8 [range 7–130] ng/L) using the ADVIA TnI-Ultra method (Laboratory Diagnostics Siemens Healthineers); undetectable cTnI values (<7 ng/L) were found in 168 individuals (24.3% of total samples). Gender-dependent cTnI values were also found in another study [47], using a prototype Access AccuTnI assay (Beckman-Coulter Diagnostics) to assess the distribution of cTnI results in a population of elderly individuals (PIVUS [Prospective Study of the Vasculature in Uppsala Seniors] study; n = 1005). At multivariable logistic regression analysis, applied to the entire study population, male gender was also independently associated with detectable cTnI levels of >6 ng/L [47].

In 2009, a gender-dependent difference in 99th URL for the highly sensitive cTnT assay (Roche Diagnostics) was also reported by Mingels et al. [48] in a reference population of 479 apparently healthy individuals; the observed 99th percentile was 8 ng/L in 215 females, and 18 ng/L in 264 males. Results from larger studies [12, 26] confirm the dependence of cTnT values on gender.

A more satisfactory definition of differences in cTnI circulating levels according to gender and age is now available thanks to the highly sensitive method using the ARCHITECT platform, able to measure biomarker values above the LoD value in the majority of healthy subjects from the day of birth to senescence [14, 15, 33, 34, 49–51]. No studies found significant gender-related differences in cTnI values of pediatric age subjects (Table 2 and Figure 2) [15, 33, 34, 50]. On the other hand, adult males usually present cTnI values that are, on average, two-fold higher than those in women; in this age group, the calculated 99th URL values are strongly gender-dependent [14, 29, 49].

Regarding the elderly, Eggers et al. [27] measured cTnI values with the highly sensitive ARCHITECT method in a cohort of 814 participants both at baseline (men age 70.2 [SD 0.2] years, 49.5% males) and after a 5-year follow-up. Even in the 382 participants who were without cardiovascular disease at the age of 75 years, the 99th percentile increased from 31.6 ng/L to 51.3 ng/L (relative increase, 62%). cTnI values were higher in males (n = 403; median: 4.1 ng/L, range 25th–75th percentiles: 3.0–6.4 ng/L) than

females (n = 411; median: 3.0 ng/L, range 25th–75th percentiles: 2.2–4.1 ng/L) [27]. Furthermore, at unadjusted logistic regression, male gender, NT-proBNP and left ventricular mass index were significantly associated with an increase in cTnI concentrations [27].

## Surrogate biomarkers

Sandoval and Apple [17] recommended an approach involving clinical history and some biomarker surrogates (including hemoglobin A<sub>1c</sub>, natriuretic peptides, serum creatinine for eGFR calculation, and cardiac imaging) for the definition of healthy individuals. In particular, Zeller et al. [29] showed that the removal of subjects with increased NT-proBNP from the reference cohort has a dramatic effect on the 99th URL value, while the effect of the addition of further exclusion clinical criteria (beyond increased levels of B-type natriuretic peptides) is negligible. Other authors reported that values of natriuretic peptides (i.e. BNP or NT-proBNP) within the normal reference range are a prerequisite for defining a normal reference population [8, 26]. Accordingly, Sandoval and Apple [17] recommended the measurement of B-type natriuretic peptides as main surrogate marker of myocardial dysfunction for defining a healthy reference population.

## Physical exercise

As is well known, not only age and gender, but also physical exercise can affect circulating cTnI and cTnT levels in apparently healthy subjects [25]. In a recent review [25], 145 studies were recovered in a systematic search. The data obtained showed that cTnI and cTnT circulating levels rise in healthy subjects not only after prolonged endurance exercise (e.g. marathon, cycling competition, mountain bike racing, ski endurance race and triathlon), but also after short-term and intermittent exercise (e.g. 30-minutes' running and basketball) [25].

Variations in cTnI and cTnT values can be related not only to the type of physical exercise (e.g. intensity and level of training), but also to blood sample timing [52]. Moreover, the method used to measure the troponin levels can affect results [25]: due to the low circulating levels of cTnI and cTnT in healthy subjects, only highly sensitive methods allow the accurate evaluation of biomarker variations in the majority of subjects tested, especially when pre- and post-exercise values are both below the 99th URL [8, 25]. Importantly, almost 100% of subjects demonstrated a significant increase in circulating levels after

exercise, especially when highly sensitive methods were employed [25, 52–56].

From a clinical viewpoint, some studies reported that increased troponin levels can be detected not only during or few minutes after the exercise, but also hours after strenuous physical effort [25, 52]. Therefore it is important to check whether each subject/patient has performed intense physical activity before cTn measurement. Troponin release after effort stress test may allow the identification of a subset of subjects at an increased risk of heart failure [57–59], and standardized physical effort with consequent cTn release variations may play an important prognostic role in patients with heart failure [57, 58].

## Biological variation

There is consensus in international guidelines [1–4, 16, 30, 32, 38] that the assessment process for diagnosing AMI includes collection of two or more blood samples for the detection of relative or absolute changes in cTn concentrations. It is widely believed that biological variations in cTnI and cTnT might affect the accurate determination of rises and falls in biomarker values after acute myocardial injury [38, 60]. As cTn has a low individuality index [60], a serial change in hs-TnI or hs-cTnI levels (using 99th percentile diagnostic cut-off) 0 to 3 h after admission may facilitate an early diagnosis of AMI [61]. Furthermore, a recent study [62] reported that biological cTnI variation is not dependent on the time interval between sample collections. These studies [61, 62] confirm that serial change in troponin concentrations is a useful tool for rapidly identifying ED patients at a low risk of ACS.

## Statistical considerations

The different approaches used for calculating the 99th URL value are a potential cause of apparent contradictions in available studies, several of which report considerable differences in the 99th URL values of cTnI and cTnT, depending on the statistical approach used for their calculation [17, 26–31, 49, 63–65]. As outliers can markedly affect the calculation of 99th URL value, the Study Group on Biomarkers in Cardiology of the Acute Cardiovascular Care Association of the European Society of Cardiology [30] recommend a sample size of at least 300 individuals per group for the calculation of

99th URL. This relatively large number is pre-requisite for ensuring a 95% probability that at least 99% of the population will have values below the highest observed troponin value [31]. According to Sandoval and Apple [17], the enrollment of more than 2000 individuals may be required to reliably establish the 99th URL value for population samples characterized for several age, gender and ethnicity sub-groups. It is therefore reasonable to consider the large majority of studies reporting 99th URL values for cTnI and cTnT statistically under-powered [17].

Parametric and non-parametric methods are usually used to calculate the 99th URL [26–29, 49, 63–69]. As they are recommended in the EP28-A3c IFCC-CLSI document [66], non-parametric methods are the more commonly used statistical approach in studies reporting the 99th URL values [17], but they do call for large samples, and the reliability of their results may be compromised by some outliers present in the database. The IFCC-CLSI document [66] suggests the robust percentile method as an alternative, especially for small cohorts. Another approach might be the use of robust iterative statistical (e.g. bootstrap) methods [67], which minimize the effect of extreme values and may also be suitable for small samples, but do necessitate collaboration from an expert statistician. However, the most important issue regarding 99th URL calculation is the identification of outlier values. The EP28-A3c IFCC-CLSI document [66] suggests that Dixon's method could be used to address this issue [69], but results obtained using other procedures, the Tukey method in particular [26, 65], have also been reported [67, 68]. Whatever the statistical approach used, the most reliable possible results will probably be obtained only by using the largest possible population groups, which should be accurately screened according to ethnic, demographic and clinical criteria [17].

The EP28-A3c IFCC-CLSI document [66] stresses that it is essential to make an assessment of interference from naturally occurring constituents in blood samples of a reference population. It is well known that interferences from, for example troponin macro-aggregate [70, 71] and heterophilic [72] or auto-antibodies [73], may produce aberrant results in troponin testing in individual samples. Finally, the EP28-A3c IFCC-CLSI document [66] recommends that all pre-analytical factors (including subjects preparation, sample collection and processing), the analytical method procedure (including lot-to-lot variation of materials and calibrators) and instrumentation must be carefully defined and used for testing the reference individuals in order to minimize the number of aberrant results.

## Mechanisms of troponin release in cardiac patients and healthy subjects

Some pathophysiological factors should be borne in mind when comparing and explaining the differences in cTn release in ischemic and non-ischemic injury in patients with cardiac disease [74–76] and that in healthy patients following physical exercise. The kinetic profile of cTnI and cTnT circulating levels after strenuous exercise in apparently healthy subjects [25, 40, 52–56, 77–90] is different from that usually observed in patients with AMI. It is well known that circulating levels of cTnI and cTnT in patients with uncomplicated type 1 AMI usually peak between 12 and 36 h from beginning of thoracic pain, gradually returning to below the 99th URL value within 5–7 days [1, 3, 40, 76]. On the contrary, in marathon runners an earlier, shorter and lower peak of cTns is usually observed. The shorter peak observed in these cases after physical exercise with respect to those occurring in AMI subjects may depend on the release of cTnI and cTnT from the cytosolic compartment, corresponding to 5%–8% of the total cTn content of cardiomyocytes [8, 75, 91]. It has been suggested that enhanced membrane permeability promoted by the production of reactive oxygen species or alterations in calcium, pH, glucose/fat metabolism, or in communication between integrins, might explain the release of cTn into circulation after strenuous exercise [25, 75]. Other authors suggest alternative mechanisms: increased cardiovascular stress, inflammation, release of troponin degradation products in “blebs”, dehydration, impaired renal clearance, and cTn expression in skeletal muscle [25], although the latter seems to occur only in the case of cTnT [92, 93].

The mechanism underlying the presence of detectable troponin levels in healthy individuals at rest is not yet well understood. cTnI and cTnT may be released due

to some physiological mechanisms related to cardiomyocyte renewal in humans [8]. Cardiomyocyte renewal primarily depends on the maturation/proliferation process of endogenous cardiac stem cells, and possibly on blood-born stem cells [94–97]. It has also been shown that the aging human heart is characterized by a gradual depletion of telocytes, which are in close contact with putative cardiac stem cells and immature cardiomyoblasts, thereby contributing to cardiac renewal [98–102].

Physiological cardiac renewal might help explain not only the variability found in relation to the age of circulating cTnI and cTnT, but also the dependence on gender in healthy subjects. The total number of cardiomyocytes renewed per day might strictly depend on myocardial mass that, in healthy men, is usually greater than in healthy women. Few available experimental data indicate that circulating cTn levels are strictly related to both ventricular mass and gender in large populations of healthy subjects [103–106]. A recent study on 4139 subjects (2099 M and 2040 F, age range 35–74 years) found that several variables, estimated by echocardiography and reflecting cardiovascular phenotypes (including left ventricular mass), were significantly correlated with cTnI concentrations in the general population, and that their distribution differed according to gender and age [105] (Table 3).

## Clinical interpretations of 99th URL values

### Are the results of cTn immunoassays biologically equivalent?

The APACE study [107] recently reported that a diagnosis other than AMI is made in about one out of five AMI

**Table 3:** Median left ventricular mass (LVM) values (25th–75th percentile, g), estimated by echocardiography, and NT-proBNP (ng/L), both variables divided according to cTnI concentrations (ng/L) measured by the high sensitive ARCHITECT method, as reported by Sinning et al. [105].

cTnI concentration	<1.9 ng/L	≥1.9–<3.5 ng/L	≥3.5–<5.2 ng/L	≥5.2 ng/L	p-Value <sup>a</sup>
LVM, g					
Women	120 (101, 142)	124 (106, 145)	130 (111, 154)	135 (116, 164)	0.0022
Men	168 (143, 194)	167 (143, 191)	175 (153, 201)	185 (158, 221)	<0.001
NT-proBNP, ng/L					
Women	69 (39, 114)	69 (38, 118)	80 (45, 142)	109 (58, 235)	0.12
Men	29 (9, 52)	26 (8, 56)	29 (10, 63)	49 (19, 130)	<0.001
Women/men ratio	523/211	665/514	509/606	343/767	

<sup>a</sup>p-Value adjusted for age.

patients if cTn is measured with another immunoassay (including highly sensitive immunoassays – ARCHITECT for cTnI and ECLIA for cTnT) using the clinical decision (i.e. 99th URL) values suggested by manufacturers [107].

From a methodological viewpoint, the finding that different cTn assays are not biologically equivalent might be explained on the basis of the different biochemical characteristics of cTnI and cTnT [4–6], the systematic differences between cTnI assays [23], and also the fact that various manufacturers use different reference populations to determine 99th URL values [108, 109]. Samples of AMI patients misdiagnosed in the APACE study [107], are probably those with cTnI and cTnT values around to 99th URL of respective immunoassay methods. These patients may be expected to be at “intermediate” risk (i.e. between the extremely low risk of individuals with cTn values near to the LoD value and the higher risk of individuals with cTn values significantly higher than 99th URL values) [110–113]. Therefore, unsurprisingly, the APACE study reported that patients with misdiagnosis of AMI with two or more cTnI assays were at an intermediate risk, evaluated by Kaplan-Meier survival curve analysis (i.e. between those with a final diagnosis of unstable angina and those with confirmed diagnosis of AMI) [107].

From the clinical viewpoint, the use of lower clinical decision values for AMI theoretically increases clinical sensitivity, but may also greatly increase the false-positive rate, decreasing the specificity of highly sensitive cTn assay. The 2015 ESC guidelines [32] propose two options for the early ruling-out of AMI using the cTnI and cTnT measurement with highly sensitive methods: (1) a single sample with cTn concentrations below the LoD, and (2) algorithms based on threshold concentrations and/or changed in concentrations measured over 1 h. Furthermore, the cost-effectiveness of diagnostic algorithms based on laboratory tests depends not only on the quality specifications and analytical performances of assay methods, but also on patient population characteristics and healthcare resources [114]. Of particular importance is the evaluation of negative and positive predictive values, as reported in the 2015 ESC guidelines [32] to support the use of algorithms for rapid ruling-out and ruling-in of AMI, which strongly depends on the demographic and clinical characteristics of ED patients [42]. However, the inclusion/exclusion criteria and the demographic and clinical characteristics of ED patient populations vary greatly from study to study [27–29, 104–109]. It is therefore not surprising that contradictory results were reported in a recent multicenter study [115] conducted to validate the performance of rapid algorithms for ruling-in or ruling-out AMI, as suggested in the 2015 ESC guidelines. The authors concluded that these rapid algorithms

could prove useful in identifying patients requiring prompt management, but that they might not be sensitive enough to allow ED physicians to confidently send patients home [115]. Therefore, we believe that clinical decision values, even if recommended by international guidelines, should be considered only indicative, and the effectiveness and efficiency of diagnostic algorithms should always be tested in routine clinical practice.

### Are gender-specific cut-offs useful for diagnosis of AMI?

The guidelines from the Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction recommend that 99th URL values should be calculated according to gender for the diagnosis of AMI [3]. However, the most recent 2015 ESC guidelines on the management of ACS in patients without persistent ST-segment elevation do not discuss this important issue [32]. Shah et al. [116] recently evaluated the diagnostic accuracy for AMI of the highly sensitive cTnI ARCHITECT assay using gender-specific diagnostic thresholds, as suggested by the manufacturer (M 34 ng/L, F 16 ng/L), in 1126 patients (46% women) with suspected ACS. The highly sensitive cTnI assay actually doubled the diagnoses of type 1 AMI [3] in women (from 11 to 22%;  $p < 0.001$ ) with respect to the less sensitive contemporary cTnI assay, but had a lesser effect in men (from 19 to 21%;  $p = 0.002$ ). Furthermore, after a 12-month follow-up, women reclassified as having AMI using the highly sensitive assay with gender specific thresholds ( $n = 56$ ) and those with AMI diagnosed using both methods ( $n = 56$ ) had the highest rate of death or re-infarction compared with women without a diagnosis of AMI (25%, 24%, and 4%, respectively;  $p \leq 0.001$ ). These data have prompted debate and research into gender-specific cut-offs for the diagnosis of AMI [117].

Mueller-Hennesen et al. [118] evaluated the impact of age- and gender-specific cut-offs (28 ng/L for  $\geq 65$  years; F 9 ng/L, M 15.5 ng/L) for high-sensitivity cTnT compared to the general cut-off for diagnosing AMI in 1282 unselected patients presenting at ED. Age-specific cut-offs led to the prognostic reclassification of patients for 3-month mortality in the entire series, and the ACS cohort (11.8% and 14.2% net reclassification improvement,  $p < 0.001$ , respectively). On the contrary, no significant differences in outcomes could be found using gender-specific cut-offs. Authors concluded that while the influence of gender-specific cTnT cut-offs on diagnostic and prognostic reclassification was only modest in patients with suspected AMI, age-specific cut-offs had a significant impact and could be considered for further validation [118].



The results reported by Shah et al. [116] and Mueller-Hennesen et al. [118] are contradictory concerning the usefulness of using gender-specific cut-off values with highly sensitive methods for cTnI and cTnT. The discrepancies between the results of these two studies may be related to some biological differences between cTnI and cTnT. However, some methodological (related to the experimental protocol and/or the statistical procedure adopted to calculate clinical decision values of study) and analytical (related to analytical sensitivity and specificity of immunoassays) issues may also play a role.

### Do differences in troponin-dependent 99th URL values depend on ethnicity?

The Multi-Ethnic Study of Atherosclerosis (MESA) study evaluated [119] right ventricular volumes and mass by means of cardiac magnetic resonance imaging (MRI) in 4204 subjects, enrolled from a population-based multiethnic sample free of clinical cardiovascular disease. Right ventricular morphology is considered an important predictor of outcome in heart and lung disease. The authors found that age, gender, and ethnic origin were associated with significant differences in right ventricular mass, volumes, and ejection fraction, their results indicating that there are not only gender, but also ethnicity dependent differences in cardiac structure and mass, thus suggesting that turnover of cTn and levels of circulating cTnI and cTnT may be affected by both gender and ethnicity.

Regarding the highly sensitive cTnI assay, results of studies investigating 99th URL values in apparently healthy individuals using the ARCHITECT method are reported in Table 4 [9, 14, 29, 120–126]. These data are from a total of 12,768 (11,237 on excluding those reported by manufacturer) apparently healthy subjects of different ethnicities, including Asian [121, 123, 125], Australian [120], European [14, 29, 122, 126] and North American [9] populations. On considering these studies overall [9, 14, 29, 120–126], median (25th–75th percentiles), the 99th URL values are 19.3 (13.5–23.7) ng/L (M 26.0 [19.6–29.5] ng/L), and (F 11.9 [10.8–18.3] ng/L). However, these studies vary greatly for population size (range, 366–2608), age of participants (age range 18–98 years), inclusion/exclusion criteria (especially in evaluating presence of asymptomatic cardiac disease and co-morbidities – e.g. hypertension, obesity, diabetes mellitus, renal disease), and statistical analysis (parametric or non-parametric) approach for calculating 99th percentile and exclusion of outliers [9, 14, 29, 120–126]. Studies following more rigorous criteria, using both laboratory and clinical data to include/exclude healthy volunteers,

tended to report lower 99th URL results for both genders [29, 120]. Although the data reported in Table 4 are significantly heterogeneous ( $I^2=99.91\%$ ;  $p\leq 0.001$ ), there was a significant difference between the 99th URL values found in men and those in women (10.97 ng/L, SE=1.979 ng/L, respectively;  $p\leq 0.0001$ ). Figure 3 shows a forest plot (calculated using the random effects model) for the 99th URL values reported by the manufacturer and clinical studies for the highly sensitive cTnI method.

Regarding the highly sensitive cTnT ECLIA method, variability among the 99th URL values reported in different studies and by manufacturers is very similar (Table 5) [9, 12, 26, 41, 124, 126–128]. Considering as a whole the clinical studies including a total of 10,078 individuals [9, 12, 26, 47, 124, 126–128], median (25th–75th percentiles) values of 99th URL results of highly sensitive cTnT ECLIA method are for the overall population 14.6 (13.2–18.0) ng/L, for men 16.0 (15.0–19.5) ng/L, and for women 12.0 (9.6–12.5) ng/L, respectively. The results reported by Gaggin et al. [128], including data related to two different populations (i.e. North American and Vietnamese cohorts) are intriguing: the 99th URL value in the Vietnamese women was higher than the respective value in men (i.e. 25 ng/L vs. 19 ng/L) (Table 5). However, the Authors reported that, as expected, in the Vietnamese cohort the median (95% CI) cTnT concentrations in men (4.0 [3.0–5.0] ng/L) were higher than in women (2.5 [2.5–4.09] ng/L). These data [128] suggest that the higher 99th URL value in Vietnamese women was probably due to outlier values not corrected at statistical analysis. Although data reported in Table 4 are significantly heterogeneous ( $I^2=99.93\%$ ;  $p<0.001$ ), there was also a significant difference between the 99th URL values in men and women for the 99th URL value of cTnT measured using the highly sensitive ECLIA method (4.59 ng/L, SE=1.5232 ng/L;  $p=0.0026$ ). Figure 4 shows a forest plot based on the random effects model for the 99th URL values reported by manufacturer and clinical studies for the highly sensitive cTnT method. Data reported in Tables 4 and 5, and Figures 3 and 4, confirm that gender-differences in 99th URL values are present for both cTnI and cTnT. In particular, on average the gender-difference between the mean 99th URL values is two-fold greater for cTnI than cTnT (i.e. 10.97 ng/L vs. 4.59 ng/L).

Given that cTnI has a lower molecular mass than cTnT (about 22,000 vs. 37,000 Da) [129], the results reported in various studies [9, 12, 26, 47, 120, 122–124] indicate that there is a larger number of circulating cTnI-related molecules than cTnT-related molecules in healthy subjects. Furthermore, since cTnI and cTnT are present in the sarcomeric complex in a molecular ratio of 1:1, these differences between the circulating cTns are probably related to differences in

**Table 4:** Values of 99th percentile URL measured by the hs-cTnI method using the ARCHITECT platform in apparently healthy adult subjects, according to literature and manufacturer data.

Gender	Number of subjects	Age range, years	99th URL, ng/L	95% CI range, ng/L	Reference
Men	766	–	34.2	28.9–39.2	Manufacturer
Women	765	–	15.6	13.8–17.5	
Overall	1531	21–75	26.2	23.3–29.7	
Men	273	19–62	36	–	Apple et al. [9]
Women	252	18–64	15	–	
Overall	525	18–64	23	16–63 <sup>a</sup>	
Men	776	–	27.0	20–67	Krintus et al. [14]
Women	993	–	11.4	10–15	
Overall	1769	19–91	19.3	14–25	
Men	1292	–	28.4	20.5–42.1	Zeller et al. [29]
Women	1316	–	12.4	9.0–20.9	
Overall	2608	35–74	21.3	17.5–29.2	
Men	246	–	14.9	–	Koerbin et al. [120]
Women	318	–	11.1	–	
Overall	564	48–95	13.6	13.2–14.6 <sup>a</sup>	
Men	597	35–65	32.7	21.1–47.9 <sup>a</sup>	Aw et al. [121]
Women	523	40–65	17.9	10.7–26.3 <sup>a</sup>	
Overall	1120	35–65	25.6	19.6–32.6 <sup>a</sup>	
Men	171	–	18.3	–	Collinson et al. [122]
Women	195	–	9.5	–	
Overall	366	45–89	12.3	6.2–21.0	
Men	349	–	25.0	12.4–30.6 <sup>a</sup>	Lee et al. [123]
Women	195	–	19.3	–	
Overall	544	20–71	19.3	13.3–28.6 <sup>a</sup>	
Men	729	–	20	14–22	Kimenai et al. [124]
Women	806	–	11	8–13	
Overall	1535	40–75	13	11–18	
Men	426	24–87	20	15–69 <sup>a</sup>	Ji et al. [125]
Women	428	18–90	19	11–41 <sup>a</sup>	
Overall	854	18–90	18	14–35 <sup>a</sup>	
Men	826	–	27.5	21.4–31.3 <sup>a</sup>	Ungerer et al. [126]
Women	526	–	21.5	21.5–101.5 <sup>a</sup>	
Overall	1352	<50	25.7	19.2–30.5 <sup>a</sup>	

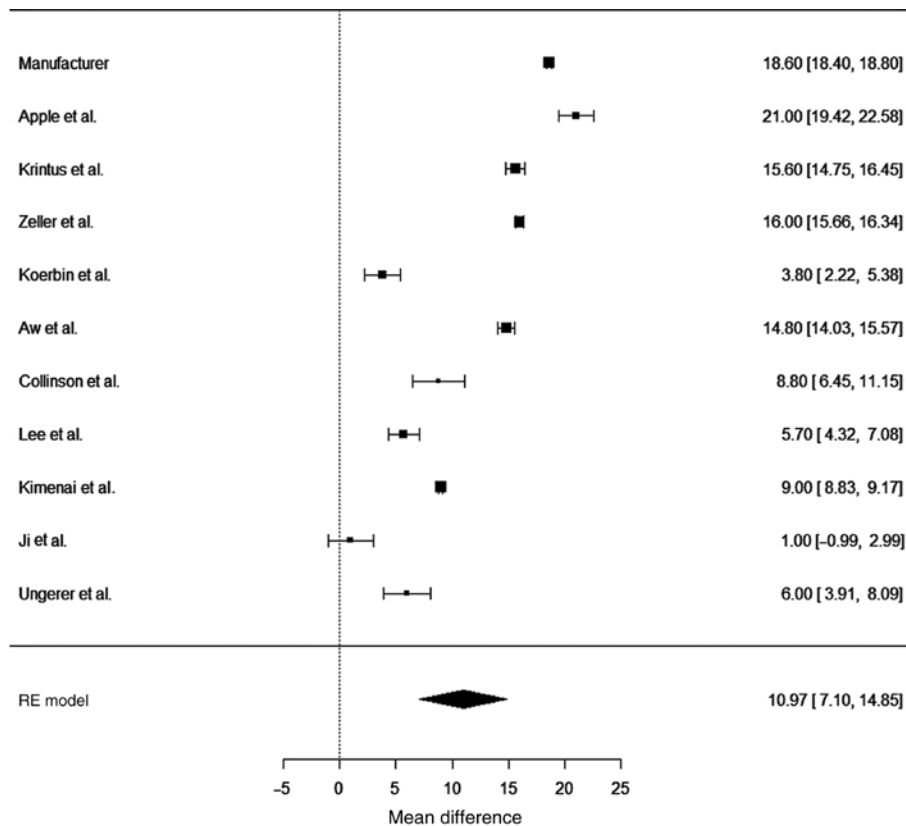
<sup>a</sup>CI 90%.

intra- [130] or extra-cellular [131–133] catabolism and the peripheral turnover of cTnI and cTnT. A recent study [134] found that circulating levels of cTnT, unlike cTnI, have a diurnal rhythm, thus bearing out the hypothesis that there are some differences in the release from cardiomyocytes and/or peripheral turnover between cTnI and cTnT.

### Clinical relevance of gender-specific decision values for cTnI and cTnT assay

From a clinical point of view, a fundamental question is whether the use of gender-specific cutoff values for highly

sensitive cTnI and/or cTnT assays actually allows a more accurate detection of patients with non-ST-segment elevation acute coronary syndromes (NSTEMI-ACS), who are at higher risk of presenting major cardiovascular events in the short term. Regarding the highly sensitive cTnI assay, some studies report that the use of gender specific decision values significantly increases AMI diagnosis especially in women, with better stratification of risk [112, 135–137], while another study found contradictory results [138]. The evidences regarding the highly sensitive cTnT assay are, on the contrary, all negative, suggesting that gender-specific clinical decision values are not particularly useful for the highly sensitive cTnT assay [117, 118, 139]. These



**Figure 3:** Forest plot showing the differences [CI 95%] between 99th URL values reported by manufacturer and clinical studies for the highly sensitive cTnI ARCHITECT method in men and women.

differences between cTns are in part expected considering the lower gender-dependence of cTnT (Table 5) compared to cTnI (Table 4) values.

As discussed in detail above, cTnI and cTnT levels in healthy adults might depend strongly on the physiological turnover of cardiomyocytes, and therefore on the cardiac mass, which is on average greater in men than in women. If so, a very close correlation would be found between the ventricular mass and circulating cTnI and cTnT levels. However, a straightforward, economical method for accurately estimating left ventricular mass (LVM) in a large population of healthy adults is currently unavailable. Echocardiographic evaluation is usually recommended for assessing LVM in large population studies and in routine clinical practice when cardiovascular risk assessment is made in asymptomatic adults [140]. A recent study [105] reported that LVM values, estimated by echocardiography, are significantly correlated with cTnI concentrations in the general population, the distribution, different in men and women, being influenced by age. Although on average men present higher values than women for cTnI and LVM than women, the same cTnI concentrations are present with similar frequency in women and men with great variations in LVM values, especially for concentrations ranging

from 1.9 ng/L to 5.2 ng/L, which cover the majority of the population (2294/4139 subjects; 55%) [105]. It is not yet known whether this overlapping is due to true differences between women and men for cardiac structure and/or troponin content, or to some limitations (including performance resolution) in echocardiographic methodology [141, 142]. In healthy individuals, echocardiography systematically shows smaller atrial and ventricular dimensions and volumes, and larger wall thickness and mass, than cardiac MRI [141]. Although cardiac MRI is considered more accurate than echocardiography in the estimation of cardiac LVM, no data are available on the correlations between cardiac mass values measured by cardiac MRI and circulating levels of cTnI and cTnT measured with highly sensitive methods in large populations of healthy adults.

From both the pathophysiological and clinical viewpoint, it is interesting to compare the circulating levels of natriuretic peptides and cTns reported in the study by Sinning et al. [105]. Table 3 reports NT-proBNP values, subdivided according to cTnI values. These data [105] confirm that circulating levels of natriuretic peptides are strongly gender-dependent, values in women of fertile age being higher than in age-matched men. Interestingly, only in men were cTnI values significantly associated with NT-proBNP

**Table 5:** Values of 99th percentile URL measured by the ECLIA hs-cTnT method in apparently healthy adult subjects, according to literature and manufacturer data.

Gender	Number of subjects	Age range, years	99th URL, ng/L	95% CI range, ng/L	Reference
Overall	533	21–71	14	12.7–24.9	Manufacturer
Men	273	19–62	20	–	Apple et al. [9]
Women	252	18–64	13	–	
Overall	525	18–64	15	13–21 <sup>a</sup>	
Men	268	–	15.5	–	Saenger et al. [12]
Women	265	–	8.9	–	
Overall	533	20–71	14.2	–	
Men	503	20–64	13.1	11.9–13.4	Franzini et al. [26]
Women	369	20–64	9.6	8.4–13.8	
Overall	872	20–64	12.4	11.5–13.2	
Men	1346	30–64	23	19–47	Gore et al. [41]
Women	1609	30–64	12	9–18	
Overall	2955	30–64	18	16–23	
Men	729	–	16	15–17	Kimenai et al. [124]
Women	806	–	12	10–14	
Overall	1535	40–75	15	13–16	
Men	826	<50	15.8	12.7–20.4 <sup>a</sup>	Ungerer et al. [126]
Women	526	<50	9.6	6.8–13.5 <sup>a</sup>	
Overall	1352	<50	14.0	10.8–17.7 <sup>a</sup>	
Men	309	–	14.5	–	Giannitsis et al. [127]
Women	307	–	10.0	–	
Overall	616	20–71	13.5	–	
Men	253	–	16.5	–	Gaggin et al. [128]
Women	312	–	12.0	–	US cohort
Overall	565	41.4(13.4) <sup>b</sup>	15.1	–	
Men	300	–	19	–	Gaggin et al. [128]
Women	292	–	25	–	Vietnamese cohort
Overall	592	41.0(14.8) <sup>b</sup>	19	–	

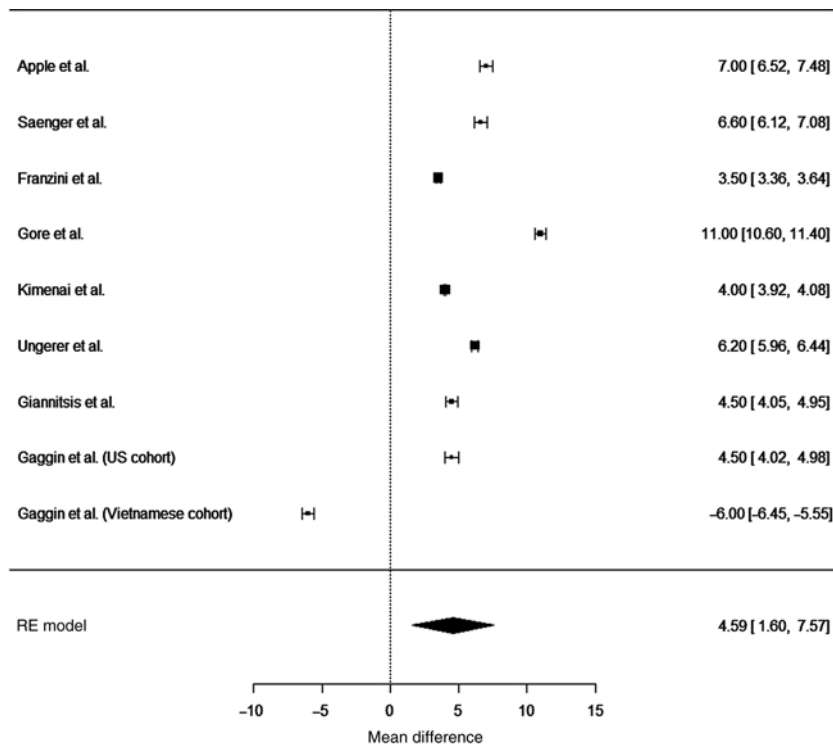
<sup>a</sup>CI 90%. <sup>b</sup>Mean (SD).

(the statistical significance found is probably due only to the group with cTnI > 5.2 ng/L). These data suggest that cardiac structural and functional changes (probably related to co-morbidities such as hypertension, diabetes and renal dysfunction), mirrored by increased NT-proBNP concentration, are present in some asymptomatic volunteers of both genders with higher cTnI concentrations (>5.2 ng/L) [143].

From a clinical viewpoint, these data confirm that natriuretic peptides and cTns provide robust additional/adjunctive/clinical information. Accordingly, these biomarkers should be always measured together in patients with asymptomatic cardiac disease for risk stratification. From a pathophysiological viewpoint, it is important to note that the opposite actions of male and female gender-steroid hormones may explain the difference in circulating levels of natriuretic peptides and cTn. Indeed, male steroid hormones may have an anabolic effects on cardiac

cells (inducing cardiac hypertrophy) [144], but also an inhibitory action on the production of cardiac natriuretic hormones [145, 146]; female gender steroid hormones may have anti-proliferative effects on myocardiocytes [144], but stimulate the production/secretion of cardiac natriuretic peptides [145, 146].

Several recent studies have demonstrated the association between the risk of major cardiovascular events and detectable, presumably still normal, cTn values measured with highly sensitive cTnI and cTnT assays [110–113, 147]. These studies give rise to the question of whether there is a myocardial injury threshold beyond which individual risk increases thus being of potential clinical utility in cardiovascular risk prediction. However, evidence from the same studies supports the hypothesis that the risk of cardiovascular increases continuously and progressively as from the limit of detection of cTn immunoassays. From a statistical



**Figure 4:** Forest plot showing the differences [CI 95%] between 99th URL values reported by manufacturer and clinical studies for the highly sensitive cTnT ECLIA method in men and women.

viewpoint, when using of decision values (e.g. 99th URL) it may be inadvisable to dichotomize continuous variables for multiple regression analyses, because this approach can significantly reduce information [148]. Therefore, the 99th URL should be used with caution in view of the risk of false positives and negatives. Clinical input is of utmost importance in minimizing this risk. Further studies are needed to evaluate whether a cut-off values for highly sensitive cTnI and cTnT assay may be effective for cardiovascular risk prediction in clinical practice.

## Future perspectives

Our critical review of data in the literature supports the observations by Sandoval and Apple [17], who reported that no uniform procedure is followed, and that no uniform guidelines have been issued by experts or regulatory bodies to ensure that researchers or manufacturers of cTn assays are guided in “their quest to define normality”. Nor is adequate attention paid to the approach for selecting a “normal” reference population and the criteria to use for calculating the 99th URL value for cTn assays. Indeed, recent studies have demonstrated the importance of the statistical manipulation of data in determining the 99th URL values, in particular regarding the use of more robust statistical

methods [26, 27, 49]. Moreover, some authors suggest the clinical relevance of determining 99th URL values using the popular cTnI and cTnT immunoassays on the same very large reference population in order to better evaluate and accurately compare the differences among methods [9]. We therefore believe that international recommendations are urgently needed, not only for demographic and physiological variables and the criteria for the definition of the healthy reference population, but also for mathematical algorithms enabling the calculation of clinical decision values. According to Sandoval and Apple [17], these guidelines should be established by an expert consensus group consisting of laboratory and clinical scientists, biomedical statisticians and industry and regulatory representatives.

Until the necessary guidelines have been developed, clinicians and clinical chemists should follow some minimal criteria for the more accurate screening of presumably normal individuals, who have a negative clinical history for cardiovascular disease, other chronic disease, or continuous use of such medication. Moreover, some surrogate biomarkers may prove useful for ruling out the presence of asymptomatic cardiac disease, such as natriuretic peptides, as well as some non-invasive cardiac investigations, such as ECG and echocardiography and carotid ultrasound [17, 26, 149–151]. Other standard laboratory tests may be useful in ruling out the presence of

renal dysfunction, anemia, diabetes mellitus, or hepatic diseases. As far as the demographic characteristics of reference population are concerned, a minimum of 300 men and 300 women are required, including several ethnic population groups, and diverse age distribution (e.g. from 18 to 70 years) [17, 30, 31].

**Acknowledgments:** The authors would like to thank Claudio Galli, MD, PhD, Associate Director, Medical Scientific Liaison Europe, Abbott Diagnostics, and Dr. Daniela Bellavista, Medical Project Manager, Medical & Market Access, Roche Diagnostics, for their information about the analytical characteristics of highly sensitive cTnI and cTnT assays.

**Author contributions:** All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

**Research funding:** None declared.

**Employment or leadership:** None declared.

**Honorarium:** None declared.

**Competing interests:** The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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