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Opinion Paper

Genetic and biochemical heterogeneity of cardiac troponins: clinical and laboratory implications

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Abstract

The contractile sarcomeric pool of skeletal muscle and heart consists of a highly-ordered arrangement of actin thin filaments, tropomyosin and proteins of the troponin complex: troponin I (TnI), troponin T (TnT) and troponin C (TnC). The cardiac myocyte expresses specific isoforms of both TnI and TnT (cTnI and cTnT, respectively), which can be distinguished from skeletal muscle isoforms by rapid and reliable immunoassays. Due to their superior cardiac specificity, cTnI and cTnT have become the recommended biomarkers for the diagnosis of myocardial injury. The most common applications are for risk stratification and diagnosis of acute coronary syndromes, albeit they can also be used for assessing a variety of other myocardial disorders. However, the excellent diagnostic efficiency of cardiac troponin testing is jeopardized by some pre-analytical issues (unsuitable specimens for testing, analyte stability, handling, transportation and storage of specimens prior to analysis), as well as by analytical concerns related to standardization or harmonization of cTnI immunoassays, imprecision at low concentrations, antibody specificity, immunoreactivity of plasma isoforms released in the blood after myocardial injury, interference from autoantibodies, heterophilic antibodies, rheumatoid factor, and human anti-mouse antibodies. In addition, although the influence of some known mutations in cTnT and cTnI on calcium sensitivity and force generation have been readily demonstrated, their influence on current immunoassays is unknown. Most mutations in these proteins may contribute to the development of certain cardiomyopathies, namely hypertrophic and restrictive. In these situations, the phenotype is characterized by the underlying diseases, and the ability to detect cTnT or cTnI is of relatively little clinical significance. However, the cTnT Arg129Lys polymorphism and those observed in the stable domain of cTnI do not significantly alter the functional properties of the molecule within the myocardium and thus are predictably asymptomatic. While the actual prevalence of these polymorphisms in the general population is still unknown, they might be a source of potential analytical problems; modifying the immunogenicity of the molecule and leading to potential false negative results. The goal of this article is to provide an overview on the potential technical and analytical challenges in the measurement of cardiac troponins, along with the significance of polymorphisms in the genes encoding for cTnI and cTnT in both health and disease.
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Keywords: cardiac markers; genetics; myocardial infarction; troponin.

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Genetics, biochemistry and biology of cardiac troponin I and T

The contractile sarcomeric pool of muscle tissue proteins consist of a highly-ordered arrangement of actin thin filaments, tropomyosin and proteins of the troponin complex. The troponin complex exists in three forms, respectively, called troponin T (TnT), troponin I (TnI), and troponin C (TnC). The repeating sarcomeric units are arranged in series, resulting in striated myofibrils (Figure 1). Each of these three subunits of the troponin complex has distinct properties and functions within the thin filaments and, working in concert with tropomyosin, regulates muscle contraction.

In humans, three TnT genes have been described on the basis of molecular cloning. These are expressed in a tissue-specific manner and encode the isoforms expressed in cardiac TnT (cTnT) muscle, slow twitch skeletal muscle and fast twitch skeletal muscle (1). Each of these genes is subject to alternative splicing, resulting in the production of multiple tissue-specific isoforms. The gene *TNNT2* is located at 1q32 and encodes a cTnT transcript of 288-amino acid, 37 kDa (Figure 1) (2). Based on the existence of a single cTnT gene, gene sequence analysis demonstrates that combinatorial alternative splicing of two 5′ exons may yield four human isoforms (cTnT-1 through cTnT-4, numbered in order of decreasing molecular size), and suggests that in the mammalian heart, combinatorial alternative splicing of these two 5′ exons is the general mechanism for producing the four common cTnT isoforms. The isoforms cTnT-1 and

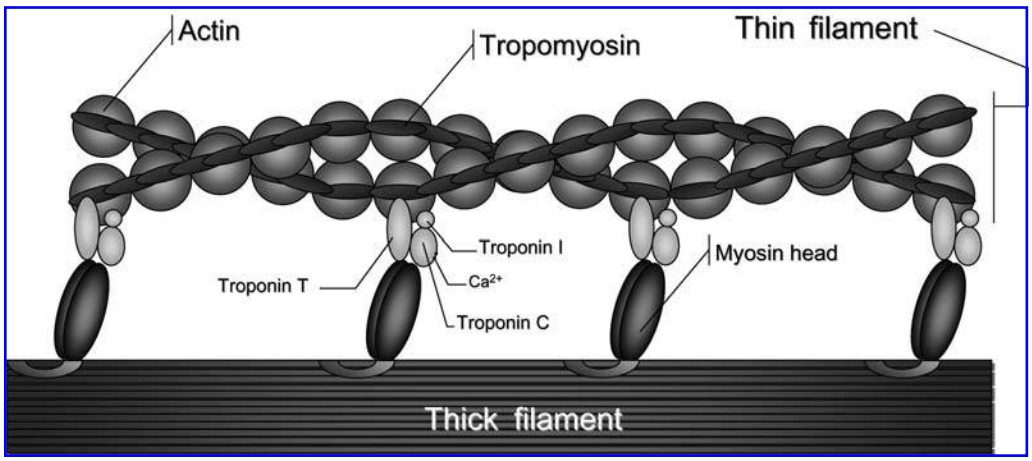

Figure 1 Structure of the sarcomeric pool.

cTnT-2, containing both peptides encoded by the 30- and 15-nt exons or the peptide encoded by the 30-nt exon alone, are expressed in the fetal heart, with cTnT-2 being expressed in very low concentrations. The cTnT-4 isoform, lacking both of these sequences, is expressed in the fetal heart and is re-expressed in the failing adult heart, whereas cTnT-3, containing the 5-residue peptide, is the dominant isoform in the adult heart (3). Thirty-one hypertrophic cardiomyopathy (HCM) mutations, six dilatative cardiomyopathy (DCM) mutations, one variant having unknown effects and five polymorphisms have been identified to date in the *TNNT2* gene (Table 1). TnT is the tropomyosin-binding subunit of the troponin complex, which is located on the thin filament of striated muscles. It binds to tropomyosin through its N-terminal tail, while its C-terminal end interacts with TnI and TnC.

The gene *TNNI3,* encoding for cTnI, is located at 19q13.4. Transcripts for this gene undergo alternative splicing resulting in many tissue-specific isoforms. However, the nature of some of these variants has not yet been elucidated. The cTnI isoform is a 210-amino acid, 22.5 kDa protein with a post-translational tail of 32 amino acids at the N-terminus (4). This sequence, along with the 42% and 45% dissimilarity with sequences of the other extra-cardiac isoforms, made it possible to develop highly specific monoclonal antibodies (MAbs) with no cross-reactivity with other non-cardiac isoforms (5). Twenty-seven HCM mutations, six restrictive cardiomyopathy (RCM) mutations, one DCM mutation, one variant with unknown effects and six polymorphisms have been presently identified in the *TNNI3* gene. TnI is the inhibitor subunit of the troponin complex, and modulates the interaction between actin and myosin. TnC is the primary Ca^{2+} buffer within the sarcomeric unit. As intracellular Ca^{2+} concentrations increase, the binding of this ion to the regulatory site of TnC causes a conformational change resulting in increased affinity of TnC for TnI. However, the affinity for actin decreases enabling the movement of tropomyosin along the actin filament which facilitates myosin-actin interaction and promotes muscle contraction (Figure 1). In contrast, in conditions of low intracellular concentrations of Ca^{2+}, the inhibitory region of TnI can bind to actin, inhibiting muscle contraction. A Ca^{2+} dependent activation of actomyosin ATPase activity also occurs due to interaction of TnT with TnC (Figure 2). In addition to the interaction sites for other regulatory proteins of the thin filament, TnT and TnI also have phosphorylation sites which can mediate contractility (6).

As previously described, TnI and TnT are present in the myocyte as specific isoforms (cTnI and cTnT) (7). Although most of the cTnI and cTnT isoforms exist as a structural myofilament-bound protein pool, there is also a free cytosolic pool within myocytes, which has been estimated to comprise 6%–8% of total cardiac troponin for cardiac cTnT, and nearly 3% of total cardiac troponin for cTnT (8). The release of intracellular proteins from injured myocardium is influenced by a variety of factors, the most important of which is their intracellular compartmentation. In contrast to the release of cytosolic proteins, the release of structurally bound proteins is slower. The release of structurally bound proteins is a two-step process, where dissociation or degradation of the subcellular structure is accompanied by a leaky plasma membrane. The initial appearance of troponins in blood following myocyte damage has been attributed to release of the unbound cytosolic pool, followed by the more prolonged release of troponins from damage to the myofilament structures. Likewise, reversible injury related to changes in the myocyte membrane are considered sufficient for the release of cardiac troponins from the free cytosolic pool. However, in cases of irreversible myocardial injury, the source of troponin release is due primarily to structural damage of myocytes (9).

The susceptibility of both cTnI and cTnT to undergo degradation by cytosolic proteases, such as the calpains, is an additional factor that can modulate the appearance of cardiac troponins in blood after myocardial damage. Since lysosomes are stable within the first 3–4 h following the onset of acute ischemia, lysosomal enzymes are not involved in early degradation of structurally bound proteins. However, depending on several conditions (e.g., time after onset of myocardial damage necrosis, size of infarc-

Table 1 Mutations in the cardiac troponin T type 3 gene (*TNNT3*).

Mutation	Exon/Intron
Hypertrophic cardiomyopathy mutations	
Phe70Leu	8
Phe77Leu	8
Ile79Asn	8
Glu83Lys	8
Val85Leu	8
Asp86Ala	8
Arg92Trp	9
Arg92Gln	9
Arg92Leu	9
Arg94Leu	9
Arg94Cys	9
Lys97Asn	9
Ala104Val	9
Phe110Ile	9
Phe110Leu	9
Phe110Val	9
Lys124Asn	9
Arg130Cys	10
Glu163Lys	11
Glu163del	11
Ser179Phe	11
Glu244Asp	14
Lys247Arg	14
Asn271Ile	15
Lys273Glu	15
IVS15+1G>A	15
Arg278Cys	16
Arg278Pro	16
Arg286Cys	16
Arg286His	16
Trp287ter	16
Dilated cardiomyopathy mutations	
Arg113Trp	9
Arg141Trp	10
Ala172Ser	11
Arg205Leu	13
Lys210del	13
Asp270Asn	15
Variants of uncertain effect	
IVS11-1G>A	
Polymorphisms	
Arg129Lys	10
Gln228Glu	13
Ser239Thr	14
Lys253Arg	14
Asn269Tyr	15

tion zone, and rate of reperfusion), the blood of patients with an acute myocardial infarction (AMI) contains variable quantities of intact cTnI and its proteolytic fragments. These can circulate in free form or complexed with the other two troponin components. As a result, the cardiac troponins are detectable in blood mainly as cTnI-cTnC (~77.5 kDa), cTnT-cTnI-TnC complexes (~114.5 kDa), with little free (22.5 kDa) or fragmented cTnI (<22.5 kDa), and free (37 kDa) or fragmented cTnT (<37 kDa) (10). Katrukha et al. showed that cTnI is rapidly cleaved by proteases in necrotic cardiac tissue; a process that leads to the rapid disappearance of the majority of intact molecules (>90%), and to accumulation of short degradation fragments. As many as 11 modified cTnI products have been reported, though their number

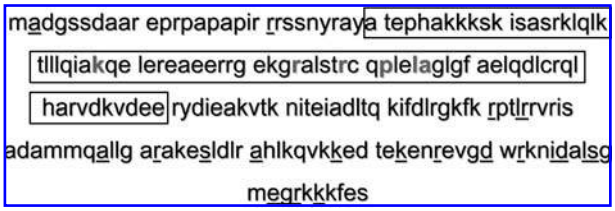

Figure 2 Amino acid sequence of the gene encoding for cardiac troponin I (*TNNI2*).
Mutations associated with inherited cardiomyopathies are underlined, variants with uncertain effect and polymorphisms are shown in gray bold. Squares represent the putative binding sites of antibodies employed in commercial immunoassays.

and extent may change over the time course following an AMI (11). The N- and C-terminal regions of cTnI are extremely unstable and easily removed. Therefore, if the epitopes of the MAbs used in sandwich immunoassays are far enough apart from each other, there is a high probability that the polypeptide chain between these epitopes will be cleaved. In agreement with this prediction, the epitopes for MAbs were located at the extreme N-terminal (14G5) and C-terminal (7F4) ends of cTnI. Thus, very small quantities of cTnI were detected using a sandwich immunoassay after a short (~8 h) incubation of tissue at 37°C. Conversely, the central region of cTnI, located between residues 30 and 110, was found to be more stable and less prone to cleavage, probably as a result of the primary structure rather than protective association with cTnC (12). cTnI might also undergo proteolytic degradation in the bloodstream, and the rate of degradation appears to depend on many different factors. Some cTnT fragments have also been detected, but these modified forms occur later. Recombinant cTnT shows essentially no susceptibility to proteolytic cleavage in normal serum. However, since patients with AMI show only a small amount of intact cTnT with one major and two minor degradation products, it is likely that these forms may be generated within the diseased myocardium itself before being released into the bloodstream (11).

Although the molecular mass seems to be of minor importance for the pattern of appearance in blood after AMI, larger molecules tend to diffuse at a slower rate, while smaller molecules may enter the vascular system to an even larger extent directly via the microvascular endothelium (9). Other factors that influence the appearance of markers in blood are the local blood or lymphatic flow, and clearance. Most proteins appear to be catabolized in organs with a high metabolic rate, such as the liver, pancreas, kidneys, and reticulo-endothelial system. Smaller molecules, such as myoglobin, also pass the glomerular membranes of the kidney and are reabsorbed and subsequently metabolized within the tubular epithelial cells. Unfortunately, however, the exact in vivo metabolism of cardiac troponins is largely still unknown.

In the remaining sections of this article, we will review the clinical significance of cardiac troponin measurement in the detection of myocardial injury,

the potential technical and analytical challenges, along with the significance of polymorphisms in genes encoding for both cTnI and cTnT.

Cardiac troponins in the diagnosis of myocardial injury

Remarkable advances in the understanding of the pathophysiology of myocardial injury over the past 20 years have allowed the identification of structural proteins and intracellular macromolecules that are released when membrane integrity fails. Proteins of the sarcomeric pool, particularly those of the troponin complex, were recognized as potentially the most effective markers. Recent international guidelines recognize five distinct types of AMI: type 1 is spontaneous due to acute plaque rupture or erosion (the typical ST elevation or non-ST elevation AMI); type 2 is secondary to an imbalance between oxygen supply and demand that may occur in patients with coronary disease and tachycardia as a result of superimposed anemia or drug overdose; type 3 is the classic AMI with verified thrombus by angiography or at autopsy in patients who have expired prior to biochemical measurement of cardiac markers or before the typical diagnostic increase has been observed; type 4 is associated with percutaneous coronary interventions (due to the procedure itself, or following stent thrombosis); type 5 is an AMI following coronary bypass graft surgery (13). According to the Universal Definition of Myocardial Infarction (14) and the guidelines of the National Academy of Clinical Biochemistry (NACB) (15), cardiac troponins have been identified as the preferred biomarkers, while creatine kinase isoenzyme MB (CK-MB) mass is the second marker of choice when troponin measurements are not available. Therefore, one troponin (either cTnI or cTnT) value that is above the decision level is required to establish the diagnosis of acute coronary syndrome (14). Both the NACB (15) and Joint Task Force for the Redefinition of Myocardial Infarction (14) guidelines recommend the use of the 99th percentile value for the diagnosis of myocardial injury in the appropriate clinical setting for the diagnosis of AMI. This recommendation is predicated on the fact that, in all the reported studies, the 99th percentile value maximizes the sensitivity, specificity and predictive value. However, since the imprecision of the troponin immunoassay at the low end could confuse clinicians, it has also been recommended that if precision is inadequate (e.g., a coefficient of variation $>10\%$ at the 99th percentile value), the troponin value at which precision is acceptable (CV $<10\%$) should be used to make the diagnosis of cardiac injury (16). The finding of a rising and/or falling pattern of troponin in blood is also important to help distinguish increased background troponin concentrations (e.g., patients with chronic renal failure) from increases in these same patients that might be due to AMI (14). Recent data confirm that such an approach might limit or eliminate the utility of rapidly rising markers, such as myoglobin (17), so that up to 80% of patients presenting with AMI and suggestive symptoms can be identified within 2–3 h (18). However, definitive exclusion of AMI might still take up to 6 h, whereas the peak values occur at 18–24 h after onset of the symptoms (4). With the advent of novel, high-sensitive (hs) cardiac troponin assays, a paradigm shift has occurred in the detection of myocardial stress, injury, ischemia and infarction, as well as in the clinical management of patients with slightly increased serum troponin concentrations (19–21). At variance with the traditional cTnT assay (Roche Diagnostics), whose traditional cut-off is 0.03 μg/L, the 10%-CV cut-off concentration of the newer hs-cTnT immunoassay from Roche Diagnostics is as low as 0.009 μg/L. This cut-off threshold value is even lower than the 99th-percentile value for the reference population (0.016 μg/L, diagnostic cut-off) (22). Similarly, the 10%-CV concentration of a new hs-cTnI assay (Hs-cTnI assay, Beckman Coulter) is as low as 8.66 ng/L, with the 99th percentile estimates for lithium heparin, serum, and EDTA plasma of 9.20, 8.00, and 8.60 ng/L, respectively (the 99th percentile limit of the traditional Beckman Coulter AccuTnI assay is 0.04 μg/L) (23). Besides playing a crucial role in the diagnosis of AMI, cardiac troponins are also useful for risk stratification in patients with angina, enable aggressive intervention with angioplasty or thrombolytic therapy and allow triage of patients with suspected AMI but without definitive clinical findings (24–26). In patients with ST-segment elevation, a positive troponin on admission identifies a group of patients (treated with either thrombolytic or mechanical recanalization therapy) having approximately a 3-fold higher risk of mortality. This increased risk has been attributed to the presence of a more severe degree of coronary artery disease, worse left ventricular function, and less efficient microvascular reperfusion. A positive troponin value on admission in patients with angina at rest also identifies a subgroup having approximately a 3-fold higher rate of cardiac events. Thus, these patients might benefit from more aggressive triage, with low molecular weight heparin and fibrinogen receptor antagonists. Finally, troponin-positive, low-risk chest pain patients on admission (i.e., no rest angina, no ECG-changes) have a 2-fold higher rate of cardiac events, although the appropriate treatment strategy remains to be determined in this clinical setting (4, 25).

A foremost aspect in the efficient translation of the biology of cardiac troponins from the bench to the bedside is the acknowledgement that an increase in cardiac troponins in blood is suggestive for the presence of, but not the underlying reason for, myocardial injury. Thus, besides AMI, there is a myriad of other diseases that can potentially cause various degrees of troponin release from the myocardium, including cardiac contusion (ablation, pacing, cardioversion, endomyocardial biopsy), myocarditis (infective, autoimmune, toxic) or myocardial extension of pericarditis, tachy-arrhythmias or bradyarrhythmias, heart block, congestive heart failure, HCM, infiltrative diseases (e.g., amyloidosis, hemochromatosis, sarcoid-

osis, scleroderma), apical ballooning syndrome, rhabdomyolysis with cardiac injury, aortic dissection, aortic valve disease, sepsis, venous thromboembolism, acute neurological disease, (including stroke and subarachnoid hemorrhages), end-stage renal disease, sickle cell crisis, and others (6, 16, 27). Therefore, an increased concentration of cardiac troponins without clinical evidence of myocardial ischemia should prompt clinicians to investigate other causes of myocardial damage. In fact, while it is unlikely that increases in plasma troponin following physical exercise always reflect clinically threatening myocardial injury, a different pathophysiological process might be advocated, involving increased cellular permeability and early troponin release (''leakage'') from the cytosolic pool, or from another readily accessible cell pool into the bloodstream (21, 28–31). Although this other pool of measurable troponin underlies an impairment in myocardial integrity and would probably improve and expedite the diagnostic process for AMI, its introduction into clinical practice is still a dilemma because there is no current evidence supporting therapeutic intervention in these circumstances (20).

Cardiomyopathies and cardiac troponins

In recent years, genomic medicine has moved from the bench to the bedside throughout all medical disciplines, including cardiology. Cardiomyopathies are defined as diseases of the myocardium associated with cardiac dysfunction. Over the past two decades, molecular genetic analysis has revealed that mutations in a variety of genes are associated with idiopathic cardiomyopathies. This information has enabled speculation about the pathogenesis of these heterogeneous cardiac disorders. Alterations in gene expression are a typical feature of cardiac dysfunction. Thus, HCM and ~50% of idiopathic DCM are familial diseases, with an autosomal dominant pattern of inheritance. It is not surprising that degradation, mutation, truncation or deficiency of cardiac troponins may affect myofilament function. Both experimental and clinical studies have shown a strong relationship between altered expression of cardiac troponins and cardiac dysfunction, including diastolic dysfunction, damage due to cardiac dilation and cardiomyopathies (32). Moreover, a variety of mutations in the genes encoding for cardiac troponins have been found to be associated with cardiomyopathies, including HCM, DCM and RCM (33).

HCM affects ~1 in 500 individuals and is typically characterized by left and/or right ventricular hypertrophy. The hypertrophy is usually asymmetric and may involve the interventricular septum, whereas left ventricular volume is generally normal or reduced. The characteristic morphological changes include myocyte hypertrophy and disarray in the adjacent area of increased loose connective tissue. Arrhythmias and premature sudden death are commonplace (34–36), especially in young adults and competitive athletes (37). Familial disease is predominant and is characterized by autosomal dominant inheritance due to mutations in sarcomeric contractile protein genes (34, 35). Up to 10 genes and over 700 mutations have been associated with this autosomal-dominant disorder. Although no genetic abnormalities can be identified in nearly one-third of these patients, mutations in the genes encoding for cardiac myosin-binding protein C (*MYBPC3*) and β-myosin heavy chain (*MYH7*) account for the majority of mutations that have been identified (20%–40% and 20%–50%, respectively). Additional gene mutations have been observed in *TNNT2* (5%–20%), *TNNI3* (~4%), α-tropomiosyn (<5%), myosin ventricular regulatory light chain 2 (*MYL2*) (2.5%), cardiac actin (<1%) and myosin ventricular essential light chain 1 (*MYL3*) (<0.5%) genes (38, 39). A variety of cardiac troponin gene defects (missense mutations, small deletions and mutations in splice signals) can cause HCM. Watkins et al. assessed the association between genetic polymorphisms and HCM in 70 families. They concluded that ~30% of cases are caused by mutations in the *MYH7*, ~15% by mutations in the *TNNT2,* and <3% by mutations in the α-tropomyosin gene. The clinical phenotype of *TNTT2* mutations was characterized by a poor prognosis and high incidence of sudden cardiac death (40). In the study by Sakata et al., cardiac troponin genes mutations were identified in 19% of the patients, 8% with *TNNT2* gene mutations (Arg92Trp and Phe110Ile), and 11% with *TNNI3* gene mutations (all Lys183Del) (41). A much lower prevalence of *TNNT2* mutations (3.3%) was described by Torricelli et al., who observed that both the clinical course and the morphologic expression of HCM caused by these mutations were very heterogeneous, since only individuals in a family with the deletion ΔGlu160 showed a clear history of sudden cardiac death; all other patients had a negative family and personal history of cardiac arrest during follow-up and the clinical course was generally more favorable (42). Unusual patterns of hypertrophy, including predominant apical involvement have been reported with some *TNNI3* defects. Based on this morphologic pattern of disease, *TNNI3* gene mutations may be more common in populations showing a high incidence of apical HCM, as has been reported in the Japanese population (43).

Although the incidence of DCM has been estimated to be between 0.4% and 2% in the community, it represents a frequent cause of heart failure leading to cardiac transplantation in young adults (44). The annual cardiac death rate in the group of people suffering from heart failure, independent of its etiology, averages 10%, but depending on hemodynamic conditions can increase to 12% of patients in class II of the New York Heart Association (NYHA) Functional Classification, and up to 56% of patients with NYHA class IV (44). Dilatation and impaired contraction of cardiac ventricles are hallmarks of this severe pathology. In the majority of patients there is no definitive evidence for a monogenic etiology of the disorder (sporadic DCM), and cases are traditionally classified

as ''idiopathic'' in the lack of other recognizable etiological factors (45). In the remaining cases, the etiology is typically multifactorial since many different clinical conditions can interact leading to the appearance of the clinical phenotype. These include idiopathic and inherited forms, as well as forms due to viral, autoimmune, alcoholic/toxic, or associated with known cardiovascular disease, where the degree of myocardial dysfunction is not explained by abnormal loading conditions or the extent of ischemic damage. Thus, it can be concluded that DCM commonly results from interactions between genetic and environmental factors, whereas ''pure'' genetic forms are rather rare (45). Heart failure is the predominant clinical manifestation and it is often progressive, although arrhythmias, thromboembolism, and sudden cardiac death are common and may occur at any stage (35, 36). The genetic background of DCM is relatively heterogeneous, and disease-associated mutations involve primarily single families with only few affected patients. Recent studies suggest that familial disease/genetic inheritance account for 30%–50% of cases, with prevailing autosomal dominant transmission, followed by recessive, X-linked, and mitochondrial inheritance. As such, several disease-associated mutations have been detected; mostly in genes encoding sarcomere, cytoskeletal, and nuclear proteins, as well as proteins involved with regulation of calcium metabolism (46). The latter includes desmin, tafazzin, δ-sarcoglycan, dystrophin, metavinculin, and nuclear envelope proteins, such as emerin and lamin A/C, *MYBPC3*, *MYH7*, *TNNT2*, *TNNI3*, α-tropomyosin, and cardiac actin (39). Mogensen et al. described 10 prevalent mutations in families with autosomal dominant DCM, the putative genes being those encoding for α-cardiac actin (*ACTC*), α-tropomyosin (*TPM1*), *TNNT2*, *MYH7*, titin (*TTN*) and *TNNI3*. However, DCM related mutations have been found in all genes encoding for proteins of the troponin complex; the overall frequency of mutations is ~6% and can predispose to malignant disease expression. Approximately 71% of carriers with the mutation experienced premature cardiac death or received cardiac transplantation mostly by the fourth decade, with an average duration of 6 months from diagnosis to event (47). Mutations in the *TNNT2* gene are the most frequent (48). Mutations of the troponin genes associated with DCM display functional characteristics that are distinct from those causing HCM. DCM-associated mutations are associated with slightly decreased Ca^{2+} sensitivity and a decreased actomyosin ATPase rate (48).

RCM is a debilitating disease, characterized by restrictive filling and impaired diastolic volume of either or both ventricles, with normal or near-normal systolic function and wall thickness. Increased interstitial fibrosis may also be present. The disease may either be idiopathic or secondary to amyloidosis, endomyocardial disease with or without hypereosinophilia, and is associated with increased risk of sudden cardiac death (35). Some case reports described familial RCM in humans, encompassing both autosomal dominant and autosomal recessive patterns of inheritance. This may result occasionally from *TNNI3* gene mutations. Thus, it has been speculated that RCM might be part of the spectrum of hereditary sarcomeric contractile protein disease (49). Whereas Kaski et al. identified sarcomeric protein gene mutations in 33% of RCM patients (17% with mutations in *TNNI3*, and 8% in each of the *TNNT2* and *ACTC* genes) (50), Rai et al. did not find any mutations in 17 Indian patients with idiopathic RCM (51). After systematic evaluation of 15 HCM patients with the restrictive phenotype, Kubo et al. detected four patients with mutations in the β-myosin heavy chain, and four with mutations in *TNNI3* (52). Mogensen et al. identified *TNNI3* mutations in six out of nine RCM patients; all these mutations appeared in conserved and functionally important domains of the gene (47). A deletion of one single nucleotide in exon 7 of *TNNI3* (nt4762delG) was also detected by Kostareva et al. (53). This deletion causes a frame shift in codon 168, introducing a premature stop codon at position 176 leading to truncation of the major C-terminal portion of cTnI (53).

Pre-analytical and analytical issues in the measurement of cardiac troponins

The release of cardiac troponins has not been attributed to tissue sources other than myocardium. Detection of cardiac troponins in blood is indicative of heart injury, except for rare analytical false positives (54). Quality specifications describing the pre-analytical, analytical and post-analytical performance of troponin assays are important for both manufacturers and clinical laboratories. Pre-analytical requirements refer not only to the sample type required for analysis and analyte stability, but also to proper handling, transportation and storage of specimens (55). The leading issues include analytical sensitivity and imprecision at low concentrations, antibody specificity and immunoreactivity of plasma isoforms (as mentioned previously, cardiac troponins are released into the blood as a heterogeneous mixture of molecular species, including intact proteins and a spectrum of modified products) (56), assay specificity, and false positive and false negative interferences (55). Although only one assay is available for measurement of cTnT, several companies market assays for measurement of cTnI. These assays frequently use different standards and antibodies with different epitope specificities. As a result, standardization of cTnI measurements is an additional concern which requires a complete reference measurement system, including a primary reference material comprised of purified analyte, a matrix-appropriate (serum-based) secondary reference material, and a reference measurement procedure that can be used to assign a value to the secondary reference material and to evaluate the analytical performance of the field methods (57, 58). Although the current second generation cTnI and fourth generation cTnT assays generally show an imprecision (CV) of ~20% at the 99th percentile of the reference population, greater than the recommended

imprecision of 10%, the new generation of hs-troponin assays allow the upper reference limits to be set ~10–100-fold lower, thereby enhancing both precision and sensitivity of the assay (55).

The current requirements for a designated reference measurement procedure for cardiac cTnI established by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) ''Working Group on Standardization of Troponin I'' encompasses a non-commercial sandwich-based non-competitive immunoassay based on use of MAbs directed against epitopes that can be considered ''pristine'' due to lack of stereochemical inhibition of binding, comparable epitope-specificity with the previous generation of commercial assays, calibration with NIST SRM 2921, and a thorough definition of assay characteristics including antibody specificity, immunoreactivity to forms of cTnI present in serum, detection limit and measurement uncertainty (58). Analytical approaches used in the ongoing characterization of the putative antibodies to be used in the reference immunoassay include multiplexed bead arrays and hydrogen/deuterium exchange coupled to high resolution mass spectrometry for epitope mapping, in conjunction with high resolution mass spectrometry of the cTnI antigen captured by the immobilized antibodies. Thus, the optimal capture and detection antibodies will be chosen to develop a robust and well-characterized immunoassay method for cTnI to be used as candidate reference measurement procedure (59). For commercial immunoassays, although methods can produce similar results, these may be far from traceability and biased significantly in terms of trueness. Therefore, standardization rather than harmonization of cTnI measurements should be the goal, whenever possible (58). Although there has been substantial improvements in harmonization of cTnI assays using the SRM2921 reference material, differences still exist that have been primarily attributed to: (a) variation in immunoassay detection methods, (b) heterogeneous forms of cTnI present in the bloodstream following cardiac injury which may influence epitope-binding sites when cTnI is oxidized, reduced, phosphorylated in either free, binary or ternary complexes, and (c) different antibodies used in the commercial assays that show different sensitivities to these modified forms of cTnI and contribute to the bias seen when using different immunoassay systems (60).

A problem in developing immunoassays, especially for measuring cTnI, is that the predominant fraction of cardiac troponins released into the bloodstream from necrotic tissue is not the intact form, but a heterogeneous mixture of proteolytic fragments and their complexes. Therefore, the antibodies used in diagnostic immunoassays should recognize not only intact free molecules and complexes comprised of free molecules with other troponin components, but also the proteolytic fragments and their complexes (61). For many years the N-terminal region of cTnI (the first 32 residues) was considered to be the best site for antibody production because this sequence of amino acids is not present in the skeletal isoform of TnI. This region is highly immunogenic and absolutely specific for the cardiac isoform. However, this region, as well as the extreme C-terminal end, are cleaved rapidly during endogenous proteolysis. As a result, sandwich immunoassays utilizing one antibody specific to the extreme N-terminal end of cTnI and another antibody recognizing residues in the central or C-terminal regions will display lower sensitivity, especially in cases of a late diagnosis of AMI when compared with assays that utilize antibodies that recognize the stable region of the protein (61). Some commercially available cTnI assays are also affected by potential oxidation of SH-groups. It is not known whether circulating cTnI occurs in the oxidized or reduced state. Thus, the effect of this phenomenon is unknown, but will, in theory, affect assay performance (10).

Falsely increased or decreased troponin results may occur because of a variety of interferences with the antigen-antibody reaction. These interferences include autoantibodies, heterophilic antibodies, rheumatoid factors, and human anti-mouse antibodies which can mimic troponin by linking the capture and detector antibodies (62). Circulating autoantibodies against cardiac troponins can give false-negative results (very low to undetectable concentrations), especially when using commercial antibodies against certain mid-fragment epitopes of cTnI in two-site immunoassays. These autoantibodies, usually represented by human or murine IgG, seem to block the binding of monoclonal or polyclonal anti-cTnI antibodies to amino acid residues 41–91 (63, 64) which constitutes a substantial part of the stable region (amino acid residues 30–110) that has been recommended as the target for cTnI assays (65). Evidence was provided that interference might also originate from circulating autoantibodies against cTnT (66), and the whole troponin complex (67). Given that the prevalence of autoantibodies against cTnI is as high as 3.2% in patients with chest pain, 3.5% in patients with non-cardiac symptoms (63), and 12.7% in healthy blood donors (68), while the prevalence of autoantibodies against cTnT is reportedly ~10% in healthy blood donors (66), this analytical interference may be a serious diagnostic challenge when one considers the importance of troponin measurements for the diagnosis of myocardial injury. The presence of heterophilic antibodies is another potential source of variable interference (from $<1\%$ to $>40\%$ in sandwich monoclonal immunoassays) and false-positive results due to falsely increased cTnI (69) and cTnT (70, 71) concentrations. The degree of interference is not easily predictable since it is characterized by the concentration (titer) of the autoantibodies, their binding specificity (mono- or polyspecific), and their affinity (with variable on- and off-rate constants). Their inhibiting effect on the formation of the two-site immunoassay complex is also dependent on the amount of the immunoreagents used. The final effect is not an all-or-none or irreversible situation, but results from at least two competing reactions that follow the law

of mass action. As such, it is predictable that autoantibodies will have their highest inhibiting effect early during the release of troponins from injured myocytes. As the release of troponin continues, the inhibiting effect will be gradually overcome (72).

The urgency for obtaining results of cardiac biomarkers warrants the use of plasma rather than serum to reduce the overall turn around time from blood collection to result. However, significant differences were reported when measuring cardiac troponins in serum or plasma with some analytical systems (10, 73). The binding of heparin to cardiac troponins may alter its immunoreactivity to various degrees depending on the assay epitopes and the heparin concentration in sample tubes. However, EDTA splits the calcium-dependent I-T-C and I-C troponin complexes, thereby decreasing the measured concentrations in troponin assays that preferentially measure these molecular forms (73). Accordingly, Uettwiller-Geiger et al. showed that cTnI values might be 4% lower in serum and 14% lower in EDTA plasma than in lithium heparin plasma, independently of cTnI concentration measured with the Access® AccuTnITM assay (Beckman Coulter) (74). Kazmierczak et al. recently showed that interference should be highly suspected in serum specimens when the initially measured cTnI concentrations was in the range of 2.0–25.0 μg/L with certain assays (71). This interference has been attributed to fibrin, although the observation of no interference in specimens with measured troponin concentration >25 μg/L led the authors to conclude that the effect of fibrin might be generally insufficient to cause spurious elevations of cTnI into this range.

Potential diagnostic implications of cardiac troponin genetic heterogeneity

An important issue which should be thoughtfully acknowledged by manufacturers is the standardization of the antibody epitopes used for assay development. The importance of such standardization originates from the complicated biochemical nature of cardiac troponins (75). It was recently observed that natriuretic peptide precursor B gene (*NPPB*) sequence variants may affect BNP physiology, possibly via transcriptional regulation. In particular, the –381 T>C polymorphism was associated significantly with BNP concentrations, with the model predicting 50% lower BNP in otherwise similar T/T vs. C/C subjects. An additional polymorphism (777 G>A – 3′ flanking region) was of borderline significance (76). This is a remarkable finding since it is the first demonstration that genetic heterogeneity in genes encoding for cardiac markers might significantly impact on their concentrations in plasma (e.g., the genotype influences the phenotype) and on clinical decision making. The effect of some of the known mutations in cTnT and cTnI on calcium sensitivity, and force generation have been clearly demonstrated, but their influence on current immunoassays is essentially unknown (10). Some mutations occurring in the epitope-binding regions of the cTnT assay have already been described. These comprise Arg129Lys and Arg130Cys which are located within the M7 MAb epitope region (residues 125–131), and the Arg141Trp mutation located within the M11.7 MAb epitope region (residues 136–147) (Table 1, Figure 1). Conversely, several mutations were identified in the stable domain of cTnI (residues 30–110): Lys58Asn, Arg74Ser, Arg79Cys, Pro82Thr, Pro82Ser, Leu85Met, Ala86Asp (Table 2, Figure 3).

Most mutations identified in both cTnT and cTnI are responsible for HCM and RCM. As a result, the phenotype is characterized by the underlying diseases and the ability to detect cTnT or cTnI is of relatively little clinical significance. However, the cTnT Arg129Lys polymorphism and those observed in the stable domain of cTnI do not materially alter the functional properties of the molecules in the myocyte and

Table 2 Mutations in the cardiac troponin I type 2 gene (*TNNI2*).

Mutation	Exon/Intron
Hypertrophic cardiomyopathy mutations	
Arg21Cys	3
Arg141Gln	7
Leu144Pro	7
Arg145Gly	7
Arg145Gln	7
Ala157Val	7
Arg162Trp	7
Arg162Gln	7
Arg162Pro	7
Ser166Phe	7
Lys178del	7
Lys183Glu	7
Lys183del	7
Arg186Gln	8
Ile195Met	8
Asp196Asn	8
Leu198Val	8
Leu198Pro	8
Ser199Gly	8
Ser199Asn	8
Glu202Gly	8
Gly203Arg	8
Gly203Ser	8
Gly203fs	8
Arg204Cys	8
Arg204His	8
Lys206Gln	8
Dilated cardiomyopathy mutations	
Ala2Val	1
Restrictive cardiomyopathy mutations	
Leu144Gln	7
Arg145Trp	7
Ala171Thr	7
Lys178Glu	7
Asp190His	8
Arg192His	8
Variants of uncertain effect	
Pro82Ser	5
Polymorphisms	
Lys58Asn	5
Arg74Ser	5
Arg79Cys	5
Pro82Thr	5
Leu85Met	5
Ala86Asp	5

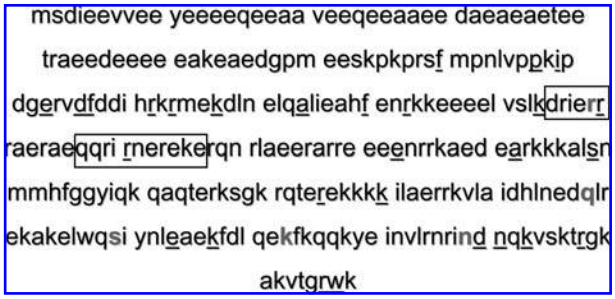

Figure 3 Amino acid sequence of the gene encoding for cardiac troponin T (*TNNT3*).
Mutations associated with inherited cardiomyopathies are underlined, variants with uncertain effect and polymorphisms are shown in gray bold. Squares represent the putative binding sites of antibodies employed in commercial immunoassays.

are thereby predictably asymptomatic. Although the actual prevalence of most of these polymorphisms is unknown in the general population (data are only available for the cTnI Pro82Ser which has a prevalence of 3%) (77), they have all been detected in healthy subjects (78). Therefore, they represent a potential source of analytical problems due to potential epitope mismatch or missing epitopes. As a result, commercial immunoassays might generate false negative results for measurement of cardiac troponins in patients that carry these mutations. According to available data, the diagnostic sensitivity of cardiac troponins for AMI throughout an extended follow-up (6–24 h after admission) to enable complete ''positivization'' of the markers, approximates, but never reaches 100%. The diagnostic sensitivity is 50%–92% with traditional immunoassays (79–81), and ~94% when using the newer hs-immunoassays (82, 83). This suboptimal sensitivity has been partly explained by the number and time of sampling in the first 48 h following the onset of symptoms, as well as due to the heterogeneity of methods and decision thresholds. It is important to mention that a larger AMI can be detected earlier and more easily than a smaller AMI, especially using the latest generation of hs-cTnI and -cTnT assays.

Besides pre-analytical or analytical problems, further clinical studies should be devised to assess whether genetic polymorphisms in the genes encoding for cTnT and cTnI might be responsible for this modest, but still clinically meaningful diagnostic inaccuracy. Novel cTnI assays, based on antibody combinations different from the conventional mid-fragment antibody approach (captures antibodies detect epitopes at amino acids 41–49 and after amino acid residue 110 in the C-terminal region, whereas detection antibodies only recognize epitopes after amino acid 110 in the C-terminal region of cTnI) (84) might offer improved detection of cTnI, especially in samples containing troponin autoantibodies. Although they still might be sensitive to the numerous mutations and polymorphisms after residue 110 (26 mutations causing HCM and 6 mutations causing RCM), predictably, they are not sensitive to the asymptomatic polymorphisms occurring in the stable domain of the molecule. However, this approach is in contrast with the current recommendations of the IFCC Scientific Division Committee on Standardization of Markers of Cardiac Damage. The committee recommendations suggest that antibodies used for the development of reliable cardiac troponin assays should preferably recognize epitopes that are located in the stable part of the molecule, and are not affected by complex formation and other in vivo modifications (65). Since the selection of the reference population strongly influences the determination of the 99th percentile, even among apparently healthy individuals (85, 86), these apparently innocent polymorphisms might significantly bias the definition of the reference ranges. This raises the question of whether genetic analysis might be advisable for establishing diagnostic cut-offs.

Conclusions

Missed diagnosis of AMI is associated with adverse clinical and economical outcomes, with more money recovered in malpractice suits than any other condition. According to the recent report of the American Heart Association (AHA) in conjunction with the Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH), an estimated 785,000 Americans suffer from a new coronary attack, and ~470,000 will have a recurrent attack in 2009. It is also estimated that an additional 195,000 silent first myocardial infarctions occur each year (87). Considering the ameliorable diagnostic sensitivity of troponin testing (e.g., 94% with the new hs-troponin assays), nearly 50,000 patients with acute coronary syndrome might be misdiagnosed in the US in 2009, when the diagnosis is based on these markers. Cardiac troponin testing is currently considered the mainstay of the diagnostic approach for acute coronary syndromes and several disease states that can damage the myocardium. In addition to the well recognized preanalytical and analytical issues, the genetic and biochemical heterogeneity of human cardiac troponins deserve further investigation, along with the prevalence of cTnI and cTnT asymptomatic polymorphisms in the general healthy population for establishing their potential impact on the diagnostic performance of commercial immunoassays for detecting myocardial injury.

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