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The continuing evolution of cardiac troponin I biomarker analysis: from protein to proteoform

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Abstract

Introduction: The troponin complex consists of three proteins that fundamentally couple excitation with contraction. Circulating cardiac-specific Troponin I (cTnI) serves as diagnostic biomarker tools for risk stratification of acute coronary syndromes and acute myocardial infarction (MI). Within the heart, cTnI oscillates between inactive and active conformations to either block or disinhibit actinomyosin formation. This molecular mechanism is fine-tuned through extensive protein modifications whose profiles are maladaptively altered with co-morbidities including hypertrophic cardiomyopathy, diabetes, and heart failure. Technological advances in analytical platforms over the last decade enable routine baseline cTnI analysis in patients without cardiovascular complications, and hold potential to expand cTnI readouts that include modified cTnI proteoforms.

Areas covered: This review covers the current state, advances, and prospects of analytical platforms that now enable routine baseline cTnI analysis in patients. In parallel, improved mass spectrometry instrumentation and workflows already reveal an array of modified cTnI proteoforms with promising diagnostic implications.

Expert commentary: New analytical capabilities provide clinicians and researchers with an opportunity to address important questions surrounding circulating cTnI in the improved diagnosis of specific patient cohorts. These techniques also hold considerable promise for new predictive and prescriptive applications for individualized profiling and improve patient care.

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Declaration of interest

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Keywords

cardiac Troponin I (cTnI); posttranslational modifications; clinical diagnosis; Mass spectrometry (MS)

1. Introduction

Troponins are proteins that directly relay myocyte excitation to contraction. As such, their expression is by and large restricted to cardiac and skeletal myocytes. The existence of cardiac-specific troponin isoforms (cTnT and cTnI) that are released into circulation upon cardiomyocyte injury have endowed these proteins with their clinical utility as plasma-based diagnostic biomarkers of cardiac damage and acute myocardial infarction (MI) [1–4]. Low-level fluctuations of circulating cTnI and cTnT are increasingly recognized as features in other diseases including heart failure and chronic kidney disease, and may therefore serve to indicate minor cardiac damage, myocyte turnover, or impaired plasma clearance. Finally, the central role that cTnI plays in molecular fine-tuning contractility as well as emerging evidence for its disease-induced modifications underscores its potential as a more accurate diagnostic tool with the potential for predictive and prescriptive applications that will further usher in the era of personalized precision medicine. The troponin protein complex consists of three individually encoded proteins, TnT, TnI, and TnC, that operate in concert to fundamentally regulate myofilament contractility in striated muscle. Each protein within the troponin complex fulfills a specific role in regulating interactions and cooperativity of myosin with critical actin-tropomyosin binding domains. Briefly, TnT anchors the position of tropomyosin on the actin thin filament. The TnC subunit functions as a direct Ca^{2+} sensor, with which it stabilizes the active confirmation of TnI to disinhibit actin-myosin contractile interactions [5]. Thus, diastolic relaxation in the myocardium occurs when cTnI maintains tropomyosin stability by binding actin and impeding access to its myosin-binding site. Systolic contraction ensues when Ca^{2+} influx evokes a conformational shift in the troponin complex that releases cTnI from actin to liberate its myosin-binding site and facilitate crossbridge cycling and force development [6]. While the fundamentals of this phenomenon occur in both skeletal and cardiac muscle, the cardiac isoforms of both TnT and TnI are expressed from distinct genes. The cardiac isoform of TnI is distinguishable from its skeletal counterpart due to an additional thirty-one Nterminal amino acids. Developing tissues express a more diverse array of proteins than fully developed tissues, and re-expression of dormant genes can occur under conditions such as wound healing or disease states. Nevertheless, cTnI expression is neither detected in developing nor adult skeletal muscle samples, and is uniquely expressed cardiac tissue [7]. Thus, degenerative muscle disease or muscle development can be excluded as a source of cTnI in patient circulation [8]. In this way, the cardiac trimeric troponin complex represents a distinct central unit that facilitates synchronous and coordinated excitation-contraction coupling in the heart.

Owing to its central role in excitation-contraction coupling, the troponin complex plays a modulating role in cardiac function. One way in which this occurs is by elevating cardiac stroke volume whereby the heart can increase its end-diastolic volume based on length-dependent myofilament activation (Frank-Sterling’s law). The ensuing increase in sarcomere

length enhances the capacity for contractile force [9,10]. This important cellular mechanism can be modulated by fine tuning myofilament Ca^{2+} sensitivity at the molecular level, in part through dynamic post-translational modifications (PTMs) to cTnI. Importantly, these modifications to cTnI and other thin and thick filament proteins collectively serve to regulate myofilament contraction and relaxation [11,12]. The counterweights to physiological cTnI modifications are maladaptive and disease-induced modifications that can adversely affect contractility. As listed in Table 1, many amino acid residues of cTnI are subject to modification, and the functional consequences of several PTMs have been determined. It is well accepted that the PTM status of cTnI in the heart can modulate heart function, thus we hypothesize that detecting and quantifying the modified forms of circulating cTnI will provide a window into cardiac disease status [13]. If this hypothesis holds, the analysis window would serve a role in tailoring therapies to individual patients. By extension, this hypothesis rests on the assumption that cTnI modifications within cardiomyocytes are faithfully reflected by those cTnI proteoforms that are found in circulation. Our lab first proposed a diagnostic potential for cTnI proteoforms in 2000, when we first showed that modified forms of cTnI were present in serum from patients with MI [13–15]. We subsequently confirmed that these modified cTnI forms in serum from patients with acute coronary syndrome [16] as well as in tissues obtained from heart biopsies [17]. Our efforts to translate these findings to a clinically relevant assay have unfortunately been hampered by limitations inherent to analytical technologies. Although we have tested many platforms [13,18,19] we are only now approaching the technical sensitivity and specificity required for quantitative analysis of total circulating cTnI, with routine analysis of modified amino acids on the horizon and the simultaneous analysis of total and modified cTnI further still.

2. Limitations and open questions in clinical cTnI biomarker analysis

The validity of applying quantitative cTnI analysis to clinical diagnostics rests on several key and open assumptions. First, the magnitude of cTnI released into circulation faithfully reflects the magnitude of cardiac tissue damage. Second, the modified forms of cTnI in circulation mirror the modified forms in cardiomyocytes. Despite overwhelming supportive clinical studies, the mechanism by which cTnI is released into the bloodstream is not fully understood. Severe cell damage associated with necrosis is generally thought to result in cTnI release, but both necrosis and apoptosis are induced by ischemia/reperfusion, and both independently result in cTnI release [20].

Circulating cTnI levels can also be elevated among healthy individuals following strenuous exercise and in disease states other than acute coronary syndromes. The underlying processes that release cTnI in these processes is also poorly understood and could skew clinical cTnI analyses [21]. Speculative mechanisms that underscore cTnI release in the absence of tissue damage include insufficient phagocytosis during apoptosis and non-pathogenic plasma membrane perforation that release cTnI from the cytosolic compartment, which corresponds to 5% - 8% of the total content of cardiomyocytes after strenuous exercise [22,23]. It is possible that the release of cTnI and its degradation products may result from exocytosis driven by increased cardiovascular stress, inflammation, and impaired renal clearance [23].

Regardless of its origin, the kinetic profile with which cTnI is released into circulation is different in patients with acute myocardial infarction (AMI) compared to marathon runners. The cTnI levels in patient with AMI peak between 12 and 36 hours, coincide with thoracic pain, and while the can remain elevated up to two weeks, they typically gradually decline below the 99th percentile within 5–7 days [21,24–26]. In contrast, circulating cTnI among marathon runners show a more acute peak with a shorter interval and overall lower peak cTnI concentrations [22,27,28]. Beyond mechanistic questions of cTnI release into circulation, several processes are additionally involved with cTnI clearance and degradation. Intact and degraded cTnI are detectable by immunoblot as early as 90 minutes after onset of symptoms with additional degradation products detectable at 165 minutes [29]. Although renal function influences cTnI clearance [30], the overall magnitude and its influence on the diagnostic accuracy of cTnI in patients with AMI remains unclear.

Although these assumptions remain at present unresolved, emerging high-sensitivity technologies already provide an opportunity to circumvent some of the inherent uncertainty and in some cases to provide answers: The ability to analyze low levels of circulating cTnI in healthy individuals can provide kinetic data that directly addresses outstanding questions about renal function. Similarly, ultrasensitive cTnI assays can provide longitudinal analyses of individual patients. Although these types of personalized metrics cannot address mechanistic questions of cTnI release, they can account for various ongoing processes that would otherwise confound the interpretation of quantitative cTnI measures. Other questions, such as the fidelity between modified cTnI proteoforms in circulation and inside cells, will fundamentally remain the subject of research hypotheses.

3. High sensitivity cTnI platforms for improved diagnostic accuracy

Timely and accurate diagnosis of MI is crucial to improving patient survival, central to assessing risk for immediate or long-term major adverse cardiac events, and could highly informative for delivering appropriate medical and interventional care. Myocyte cells undergoing injury and necrosis during MI cTnI is released into the circulation, where the protein can remain elevated for up to 7 days with a maximal elevation between 18 and 24 hours post MI [31]. The estimated levels of cTnI released after MI are on the order of 500–50,000 ng/L [32], but the absence of harmonization between assays precludes the establishment of quantitative diagnostic thresholds. Instead, 2015 European Society of Cardiology guidelines for MI propose that high sensitivity cTnI assays be used for early ruling-out of MI based on diagnostic algorithms, or for cTnI measurements falling below the limit of detection for an assay [33]. To this end, a recent diagnostic algorithm with high-sensitivity cTnI enabled patients to be safely triaged within a 1 hour timeframe [34]. Modified forms of cTnI track differentially over time, with a distinct rise and fall profiles [13,16,29,35] suggesting a need for multiple proteoform profiling to comprehensively capture and assess individual risk.

Beyond the immediacy of patient care, accurate analysis of circulating cTnI levels in the diagnosis of myocardial damage can serve to identify and triage low-risk individuals, avoiding unnecessary or inappropriate care and hospitalization, minimizing hospital stays, or providing more timely care, thus preserving medical resources [36–38]. The challenge lays

in discriminating between cTnI results that indicate low-risk and those that simply indicate low secretion. This challenge can only be addressed with improved quantitative accuracy at the low end of cTnI concentrations using high-sensitivity cTnI platforms. The International Federation of Chemistry has a record of 25 commercially available cTnI assays ranging from well-established assays to more recently developed high-sensitivity platforms [39].

Current recommended clinical guidelines call for excluding cTnI circulating concentrations below the 99th percentile, with serial measurements after 6- and 12-hours [40,41]. This convention accounts for the analytical variability and sensitivity constraints inherent to more conventional cTnI platforms. While these traditional assays were largely limited to the detection of elevated cTnI levels that almost certainly arose from AMI, immunoassays for measuring and quantifying cTnI have been improving and achieving higher sensitivity [40,41]. Modern high-sensitivity clinical assays can accurately detect cTnI at 1.1 – 0.001.9 ng/L in patient serum (Abbott Architect hs-cTnI) or with the 99th percentile placed at 8.6 ng/L (Beckman Coulter Access hs-cTnI), corresponding to a sufficient sensitivity to detect circulating cTnI in healthy individuals arising from presumptive normal low-level cardiomyocyte turnover. Beyond clinical platforms, three prominent research-grade cTnI platforms can achieve higher sensitivity, Nanosphere, Singulex and Quanterix. The Nanosphere TnI assay is a doubly amplified sandwich immunoassay that makes use of the company's Verigene nucleic acid detection protocol, but this platform is currently no longer commercially available. Singulex achieved ultra-high-sensitivity by conjugating the capture antibodies to paramagnetic particles followed by cTnI analysis using quantitative single molecule flow cytometry [42]. Finally, Quanterix initiated a "digital ELISA" platform to describe its fully automated ultra-sensitive cTnI assay, in which cTnI is sandwiched between an enzyme-labeled detection antibody and a capture antibody conjugate to magnetic beads. Beads are subsequently dispersed into femtoliter-scale wells, which are scanned on an individual basis for fluorescence. In our hands, we have used the Quanterix platform to achieve a limit of detection of 0.005 ng/L with a 99th percentile value of 7.95 (90% CI from 5.48 to 10.52 ng/L).

High-sensitivity platforms are playing an important role in describing cTnI concentrations not only to define the 99th percentile of healthy individuals, but also throughout the continuum of patients with myocardial injury. Even in the acute setting, serial measurements with high-sensitivity platforms, performed under a protracted 3 hour timeframe, were added to ESC guidelines for the management of non ST-elevated myocardial infarction in 2011. which allows for rapid rule-out of AMI [41,43,44]. Alternatively, even a 1-hour assessment can be applied to high-sensitivity cardiac troponin assays using a diagnostic algorithm to measure increasing cTnI levels [33,45]. However, the concept that cTnI levels may represent injury or natural turnover of the myocytes infers that baseline levels will differ between individuals. This raises questions concerning lower baseline levels of circulating cTnI in women compared with men, and whether baseline values increase with other patient characteristics, including age or diabetes, that might impair the regenerative capacity of the heart. High-sensitivity assays can contribute to establishing more appropriate reference ranges for such cohorts, and patients are likely to benefit from the improved accuracy [46]. Ultimately, platforms capable of reliably detecting basal cTnI values can be applied to screen patients longitudinally on an individual basis, allowing for long-term profiling of

cTnI levels to provide a “molecular window” into the status heart health. This type of personalized analysis could not only be applied in a predictive way to identify patients at risk for myocardial infarctions, but also prescriptively in the management of more chronic conditions such as heart failure, as a means of assessing the effectiveness of treatment strategies. Finally, high-sensitivity cTnI analysis platforms could hold prescriptive potential beyond the management of cardiovascular diseases by helping to define personalized dosing parameters for chemotherapeutic agents that are associated with cardiotoxic side effects.

For a cTnI assay to be considered “high-sensitivity”, it must report the levels above 99th percentile with a high degree of confidence while producing a coefficient of variation at or below 10% at the 99th percentile concentration of healthy reference population. Additionally, a high-sensitivity assay should be able to measure and report cTnI concentrations from a large proportion of the general population. Currently, that proportion is often considered to be 50%, although a value of 80% is generally accepted as more reasonable [3,47]. Moreover, the ability to accurately define baseline cTnI values across an increasingly stratified cohort of patients has initiated efforts to establish a standard reference population of healthy individuals from which to define an accepted 99th percentile along with associated reference range.

4. Age and Sex Considerations

General standard cTnI reference ranges for the baseline of healthy individuals represent an important inroad towards more precise and accurate diagnosis for patients who might otherwise not have been sufficiently captured because they can account for patient characteristics such as sex and age [48]. This is especially true of women with acute coronary symptoms, who are less frequently diagnosed with AMI as men, and may have worse outcomes as a result [49,50]. Precise reasons for this discrepancy remain unclear, but likely involve 1) a requirement for lower reference values for biomarkers of cardiac injury among women, and 2) generally lower physiological cTnI values throughout the progression of coronary artery disease in women.

Although the current lack of established reference ranges precludes sex-specific cTnI cut-off values for the diagnosis of myocardial damage, high-sensitivity and ultra-high-sensitivity cTnI assays should be superior in both accuracy and precision, and provide earlier indication of a pending MI although this is can be confusing due to the fact, other non-MI disease can have low levels. The guidelines from the Join ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infract recommends that the 99th URL values should be calculated with gender specificity [25]. Evaluation of high sensitive cTnI ARCHITECT assays using the suggested thresholds of 3.3 ng/L in men and 1.6 ng/L in women doubled the diagnosis of type 1 AMI in women with a lesser effect in men [25,51]. Mueller-Hennessen applied age- and gender-specific cut-offs to a highsensitivity cTnT assay and found no significantly different outcome compared to using a general cut-off for gender specificity [52]. Those contradictory reports on high-sensitivity cTnI and cTnT fuel an ongoing debate of the usefulness of using gender-specific cut-off values. However, the observed changes could occur due to the differences in the biology of cTnI and cTnT, and further research is needed to clarify the usefulness of a gender specific cut-off. The ability to

detect and track cTnI levels in patients prior to an infarction may help identify women with increased risk of cardiovascular events [53]. As with sex-specific reference ranges, the clinical advantage of age-specific cTnI cut off values remains unclear [53]. Certainly, the 99th percentile cut-off points for cTnI values in the general population are neither uniform across age, sex, or co-morbidities [54,55].

Despite the obvious advantage of high-sensitivity and ultra-high-sensitivity cTnI platforms, all immunobased assays suffer from the fundamental limitations of antibodies including potential quantitative variance relating to antibody affinity, and an altered affinity to modified or obscured epitopes. cTnI in myocytes can be phosphorylated, acetylated, citrullinated, truncated, oxidized or reduced, and otherwise modified prior to its secretion into circulation (see table 1). Furthermore, the common form of circulating cTnI has been proposed to reside in a TnI-TnC complex, along with tropomyosin complexes in circulation [56].On one hand, any of these alternative cTnI forms can affect antibody binding to decrease immunoassay accuracy [13,56,57], on the other, these different forms may provide deeper insights into disease mechanism and may yield new assays to diagnose cardiovascular diseases with a greater degree of precision.

While the quantitative analysis of modified cTnI proteoforms represents a challenge for immunoassays, it is well suited for targeted mass spectrometry (MS)-based assays that monitor peptide precursors and several fragment ions. Targeted MS assays can be built to simultaneously quantify truncated, proteolysis, and otherwise altered cTnI proteoforms. Unfortunately, current quantitative MS-based methods for cTnI are lower throughput, require vastly more complex preparative workflows, and lack the type of signal amplification that endows immunoassays with their sensitivity. Nevertheless, by incorporating automated immunoenrichment methods upstream of targeted MS, our group can currently achieve a detection limit of approximately 1 nmol of recombinant cTnI spiked into cTnI-depleted serum.

5. Posttranslational Modifications as compensatory mechanisms and potential biomarkers

Once translated, the cTnI protein is subject to extensive PTMs (table 1). Owing to the relative ease with which it can be analyzed, phosphorylation is the most widely studied and best characterized PTM of cTnI, while only little is known for modifications such as O-GlcNAcylation, citrullination, acetylation and oxidation. Unsurprisingly, these protein modifications often play causal or contributing roles within pathological phenotypes. In either case, an altered pattern of PTMs on cTnI may serve as a set of novel biomarkers for the phenotype with which it is associated. Already, several altered PTM patterns are known contributors or consequences of cardiac disease [58,59]. Proteolytic cleavages of cTnI correlate with myocardial stunning and ischemia/reperfusion injury [60], and mutations in the cTnI gene are associated with hypertrophic cardiomyopathies [61]. Because altered PTM profiles on myocardial proteins are associated with dysregulated cellular signaling during disease progression [62], their profiles may serve as fingerprints of cellular signaling pathway activity. Thus, PTMs have the potential to serve as ideal biomarkers, provided their

identification and quantification can be translated to clinical-grade assays [14,63]·[64]. In principle, once a PTM is identified, detection antibodies can be developed to facilitate its analysis. In practice, this approach requires substantial antibody development for each PTM individually, it is prone to antibody variability and bias, is not easily amenable to multiplexing, and requires prior knowledge and analysis of a PTM. Thus, an MS-based approach with sufficient sensitivity is ideally suited for the analysis of multiple PTMs and proteoforms. Improvements in MS technology makes it possible to reliably quantify PTMs on a large scale, and this has given rise to an exponential increase in the numbers and types of identified PTMs. As a result, the discovery of PTMs surpasses their experimental characterization, and a many modifications remain neither associated with any functional outcomes nor correlated with specific disease states [65–67]. This is similarly true for PTMs identified on cTnI (see Table 1). The effective application of PTM patterns on cTnI as biomarkers requires a deeper understanding of the cause and consequence of specific modifications culminating in a clear association with pathological conditions. The inception of this process is the ongoing characterization of modified cTnI forms with a focus on their regulatory functions.

In the human heart, cTnI phosphorylation is currently estimated to occur at a range of 1.4 – 1.8 mols-of-Pi/mol [6], although this range may increase as advances in MS instrumentation improve sensitivity. In general, the overall phosphorylation level decreases under pathological conditions such as hypertrophic cardiomyopathy (HCM) [68,69] and among patients suffering from heart failure (HF) [19,58,70–72], but this may occur reciprocally with other modifications such as O-GlcNAc, which can occur on the same amino acid residues and displace phosphorylation. To this end, O-GlcNAcylation is increased in the cardiac hypertrophy [73] and in heart failure [74,75]. During the development of heart failure, the phosphorylation pattern at various sites on cTnI becomes altered in accordance with changes in kinase and phosphatase expression or activity. Indeed, hyperphosphorylation of cTnI contributes to cardiac dysfunction in compensated myocardial hypertrophy and early stages of HF [59]. Similarly, O-GlcNAcylation at S150 has been characterized in a model of heart failure and may be associated with decreased Ca^{2+} sensitivity [74]. Furthermore, O-GlcNAcylation appears to be indispensable to the failing myocardium, suggesting that this modification may be an adaptive response [75]. Thus, an increase or decrease in phosphorylation and/or O-GlcNAcylation on specific cTnI residues could be indicative of specific pathological processes. In the absence of sufficient antibody specificity, the direct analysis of these cTnI modifications is most often accomplished from cardiomyocytes using LC-MS/MS methods with enrichment strategies to overcome inherent dynamic range constraints. Furthermore, in the case of O-GlcNAcylation, direct analysis and peptide mapping often require an additional level of preparative and analytical complexity to overcome the labile nature of the glycosidic bond.

Likewise, our group has reported elevated staining of citrullinated proteins in human myocardial samples with rheumatoid arthritis [76]. In subsequent work, we used a data-independent MS acquisition approach, with a citrullination-specific peptide ion library to identify citrullinated myofilament proteins, including cTnI, with heart failure [77]. On one hand, the staining approach can be accomplished quickly and is relatively straightforward from a technical perspective, but it cannot identify specific proteins or residues; On the other

hand, data independent acquisition MS can identify citrullination on specific peptides, but is low throughput and requires considerable expertise. Neither technique is particularly well suited for the analysis of citrullination of circulating cTnI. These types of technical challenges currently stand as major hurdles ahead of translating the characterization of cTnI modifications to a clinical tool.

A better suited candidate PTM as a clinical tool would be acetylation, since it once preserved it would be easily detectable in immune-enriched cTnI from patient samples. Acetylation of cTnI has been identified by MS approaches in guinea pig heart. Among the 8 identified acetylated residues (see table 1) two are found to have a relevance for cardiac health [78]. Mutation in K36 is associated with dilated cardiomyopathy and a mutation of K193 confers restrictive cardiomyopathy. Intriguingly, whether lysine acetylation is playing a role during the progression of the disease has not been investigated [79,80].

6. Tracking phosphorylation of circulating cTnI could reveal functional status of the heart

Extensive research efforts have been directed towards defining the cTnI amino acid residues that are subject to phosphorylation, which in turn precipitated equally extensive efforts towards defining the functional consequences of the many of the phosphorylated amino acid residues. The summary below illustrates the link between specific phosphorylated or dephosphorylated residues of cTnI and, where known, their putative functional consequences. MS-based quantification of cTnI reveals a diversity in the abundance of unphosphorylated, mono-phosphorylated, and bis-phosphorylated cTnI peptides between heart compartments. A pig model indicated that cTnI was phosphorylated to a significantly greater extent in ventricles compared to atria [81] and further WB studies revealed that S22/S23 (S23/S24 accordingly in human) phosphorylation was lower in atria. This compartmental heterogeneity may partly account for differences in Ca^{2+}-sensitivity between heart chambers.

Specific amino acid residues of cTnI are targets of multiple kinases including PKA, PKC, PKD, PKG, and AMPK. The length-dependent increase in myofilament Ca^{2+} sensitivity of cardiomyocyte can be further increased by PKA treatment, and elevated phosphorylation of cTnI [82]. PKA mediated phosphorylation of several myofilament and Ca^{2+} handling proteins is an important mediator of positive inotropic and lusitropic effects upon beta-adrenergic stimulation [83,84]. Thus, promoting cardiac pressure development by enhancing myofilament length-dependent activation and via Frank-Sterling mechanism [83,85,86]. Recent studies revealed that cTnI phosphorylation is able to regulate myofilament length-tension relationships independent of pre-load dependent length-tension [87,88].

An activated beta-adrenergic receptor pathway during stress or exercise activates PKA, leading to elevated cTnI phosphorylation [89,90] and altered Ca^{2+} sensitivity. Specifically, phosphomimetic experiments show that PKA-mediated phosphorylation of residues S23/24 lowers myofilament Ca^{2+} sensitivity and induces accelerated Ca^{2+} dissociation from TnC, which in turn discourages cTnI-cTnC interactions and promotes myocardial relaxation [91–94]. The mechanism of myocardial relaxation is important for maintaining adequate filling

during diastole and keeping actin-myofilaments at an optimal performing length [95]. Beta-adrenergic signaling along with PKA activity are impaired with end-stage HF, leading to a decrease in S23/24 phosphorylation [96], 1while less severe stages of HF are associated with undetectable levels of S23/24 phosphorylation [97]. Thus, HCM with normal systolic but impaired diastolic function (New York Heart Association class III) is associated with decreased S23/24 cTnI phosphorylation [64,98,99] and increased myofilament Ca^{2+} sensitivity [100].

In HF, PKC (isoforms alpha, beta1, and beta2) expression is increased [101] leading to higher phosphorylation of PKC specific cTnI-residues [19,102]. PKC mediated phosphorylation on cTnI S43 or S45 contributes to cardiac dysfunction in heart disease [19,103–105]. Both residues are located on the H1 alphahelix of cTnI [106]. A similar regulatory mechanism to S23/24 phosphorylation was predicted upon phosphorylation of S43/45, yielding an accelerated dissociation of Ca^{2+} from cTnC [103]. However, S43/45 phosphorylation induces additional conformational changes that have not been observed with S23/24 pseudophosphorylation. Decreased crossbridge dissociation rates are thought to cause slower sliding speed and lower myofilament shortening rates [107].

Phosphorylation at S43/45 extends the interaction of cTnI and cTnC while keeping thin filaments in an inactive state [103,108]. In isolation, this S43/45 effect could significantly alter contractility, but it is tempered by cellular adaptions via cardiac myosin binding protein-C S282 and cTnI S23/24 phosphorylation that normalize cell shortening or re-lengthening [103]. Complete replacement with cTnI phosphomimetics of PKC targets S23/24, S43/45 and T144 resulted in a modest decrease in several components of cardiac function including pressure development, rate ejection fraction, and relaxation rate. Conversely, cardiac function is substantially reduced by cTnI pseudo-phosphorylation at S43/45 and T144, or by de-phosphorylation of S23/24 [109–111]. Treatment of non-failing and failing human cardiomyocytes with the catalytic domain of PKC or with isoforms alpha and epsilon had no effect on maximal force [112,113]. Alternatively, myofilament Ca^{2+} sensitivity in animal models substantially decreases with bisphosphorylation of cTnI at S42/44 without affecting force development [114]. Collective insights provided *by in vivo* studies with elevated pS23/24 in acidosis and S43/45 phosphomimetic experiments indicated that activated compensatory mechanisms and adaptive signaling are playing a role in disease and cTnI regulation [103].

The final seventeen c-terminal amino acids of cTnI are prone to proteolysis with Ischemia/Reperfusion injury [115–117], and residue S199 within this segment can play an important regulatory role in myocardial contraction by decreasing cTnI binding to actin and actin-tropomyosin [118]. Pseudo-phosphorylation of this PKC-alpha targeted residue increases Ca^{2+}- sensitivity in a length independent manner. Interestingly, S199-mediated Ca^{2+}-s2ensitization neither augments maximal and passive isometric forces, nor the steepness and rate of Ca^{2+} force relation. Elevated phosphorylation of S199 was detected in a dog model of dyssynchronous pacing-induced HF and in endstage heart failure, while cardiac resynchronization therapy enables a reversion of pathological increased S199 phosphorylation revealing S199 as a potential therapeutic target [19], suggesting that

phosphorylation of S199 may regulate calpain I mediated proteolysis of the N-terminal cTnI region [118].

AMPK mediated cTnI phosphorylation augments myocyte contractility and increases cell shortening likely through delayed cross-bridge detachment, which leaves both the transient Ca^{2+} amplitude and ATP consumption unaffected. This pattern of phosphorylation appears to provide an energetic benefit for myofilament function and represents a potential mechanism for maintaining heart function under conditions of energetic imbalance such as heart failure [119]. AMPK induced phosphorylation of cTnI S150 increases myofilament Ca^{2+} sensitivity [85,119,120] but the effect is blunted by pseudophopshorylation of S23/24 [85,87]. Beta1 and beta2 Knock out mouse models have shown that a loss of AMPK activity can cut cTnI S150 phosphorylation by half [121]. AMPK induced myofilament phosphorylation may play a compensatory role for maladaptive creatine kinase / adenylate kinase phosphotransfer activity in early stage HF [122], where AMPK may target its kinase activity to S23/S24 or S150 cTnI.

While phosphorylation of cTnI continues to be intensely studied, it is by no means the only modification. Top-down MS workflows that use electron capture and electron transfer dissociation for intact protein analysis have identified three cTnI truncation isoforms and an acetylation on residue A2 [123]. Further investigation of acetylated, oxidized, and glycosylated cTnI residues will shed much-needed light on the regulatory impact and occurrence that this modification plays in physiological and pathological conditions.

7. Prospects of clinical cTnI Post Translational Modification Analysis

The quantitative analysis of PTM-based protein biomarkers in plasma or serum samples has three significant hurdles.

1. The need to measure the total concentration of the protein as well as the ratio between modified forms inherently requires multiple measurements per assay.
2. The broad dynamic range in plasma and serum protein concentrations [140]. The quantitative analysis of low abundant biomarkers for noninvasive clinical diagnoses requires easily accessible biofluids such as blood. Unfortunately, the mismatch between sample complexity and the protein dynamic range of blood represents a big analytical challenge. Furthermore, exceedingly low abundant modified forms of analytes including cTnI are further stoichiometrically disfavored.
3. The clinical acceptance and implementation of a “new cTnI measurement” that includes PTM information.

Since cTnI is a low abundant protein among the high range and highly concentrated protein in blood, there is a compelling need for enrichment strategies for the reliable detection of modified cTnI PTM’s in patient’s serum. As previously stated, numerous underlying methods downstream of MS analysis can be used to enrich and thus quantify PTMs, including gel-electrophoresis, affinity- or immuno-based enrichment, and protein crystallography [141]. The main challenge is to translate downstream sample enrichment

and sample processing methods to platforms that would be suitable to a clinical laboratory. Specifically, these methods must be performed on platforms that allow simplified handling, high-throughput, and with low susceptibility to variations occurring from sample processing. Automated workstations are increasingly capable of meeting these demands where sample preparation steps are concerned, and automated cTnI immuno-enrichment and sample processing methods may be capable of meeting standards for clinical diagnosis. Likewise, despite the richness and complexity of MS data, pipelines for downstream data analysis steps that yield quantitative results can also be implemented. A remaining limitation to throughput remains MS acquisition, which represents a bottleneck in that MS acquisition cannot be multiplexed. With respect to PTM's, the ratio between modified cTnI proteoforms normalized to total cTnI within a sample represents an important informative measure. Simple quantitative measurements for individual cTnI PTMs require enrichment workflows, and in the absence of total circulating cTnI values, these cannot distinguish a disease-induced PTM flux from an altered secretion profile. Ultimately, the most challenging aspect to achieving this type of ratio lays in its requirement for multiple sample preparation protocols and multiple acquisitions from a single sample. Both for technical and throughput reasons, this type of workflow remains largely relegated to the research domain.

The two main MS-based proteomics approaches are the top-down approach for intact protein analysis and the bottom-up approach for peptide analysis. In the bottom-up approach, samples are digested by proteolytic enzymes and the resulting peptides are detected by MS or tandem MS (MS/MS) [142–144]. An unambiguous absolute quantitative measure of peptides and their PTMs can be achieved under several bottom-up strategies, the most promising among these include the incorporation of Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) [145,146], and Multiple Reaction Monitoring/Mass Spectrometry (MRM/MS) with isotopically-labeled internal standards [19]. Previous studies from our lab using MRM/MS assays targeting cTnI peptides from human heart tissue sample resulted in the detection and quantitation of 14 phosphorylation sites [19]. However, more recent experiments involving the detection of similarly phosphorylated cTnI indicate the requirement for increased sensitivity in plasma and serum samples. An important limitation of the bottom-up MS approach for the quantitative analysis of modified cTnI proteoforms lays in its inherently incomplete sequence coverage. Informative modifications that reside on peptides that are inconsistently cleaved or unreliably detected is inherently lost. Furthermore, the quantification of a specific peptide is a direct function of its underlying physicochemical characteristics and is fundamentally influenced by its charge state. Finally, the abundance of a peptide is related to the its ionization efficiency and detection in MS. For these reason, the relatively low abundance of modified peptides compared to their unmodified counterparts often requires enrichment strategies. All of these limitation speak to the absolute requirement for validation each quantified peptide and PTM inherent to an MRM assay, and these parameters must be continuously assessed by integrating standard QC metrics indicative of both sample preparation and peptide targeting steps [147].

The reciprocal to peptide-level bottom-up MS is the top-down analysis of intact proteins. The advantages of intact protein analysis include its inherent ability to distinguish between different proteoforms, and to quantify putative PTMs across entire protein sequences. The proteoform distribution and insights into targeted protein modification characteristics can

potentially be used as a fingerprint that reflects the physiological status of a patient, assuming a healthy standard reference profile can be defined. In bottom-up workflows, proteins are digested and MS-based quantitation is performed from to their constituent peptides. In this scenario, distinguishing cTnI from its skeletal isoform is entirely reliant on detecting peptides that encompass the thirty-one amino acid N-terminal of cTnI [7]. Thus, depending on the peptides that are analyzed, bottom-up MS-based detection of circulating TnI can represent a composite of cardiac and skeletal isoforms, and one would expect this problem to be especially pronounced for patients with degenerative muscular disorders. Upstream antibody-based enrichment strategies that target cardiac-specific protein domains would be well suited to preserve the cardiac specificity of such MS-techniques.

Ultimately, in a similar way to high-sensitivity cTnI immunoassays, top-down MS-based cTnI analysis could be viewed as a potential tool to produce a personalized profile that tracks longitudinal changes to the cTnI fingerprint. Several top-down MS-based assays are in use for clinical diagnosis, including carbohydrate deficient transferrin for chronical alcohol abuse, and apolipoprotein C-III proteoforms for congenital disorders of glycosylation [148]. Furthermore, clinical assays relying on single reaction monitoring LC-MS have been established for intact insulin-like growth factor 1 and 2, and insulin and their proteoforms for the clinical diagnosis of growth disorders and assessing insulin resistance. PTMs of cTnI have extensively been studied in heart tissue using top-down MS to identify S22/23 phosphorylation in healthy hearts [123,149,150] as well as hyperphosphorylation of S42/44 and T143 in animal models of HF [151]. Furthermore, several degradation products were detected with top-down approaches including the three major degradation isoforms with C-terminal truncations [64,149]. By preserving protein integrity, the top-down MS approach decreases the overall number of analytes in a sample. Conversely, a peptide mixture is chemically more uniform and analytically less challenging than an intact protein mixture. For this reason, top-down approaches often rely on specific upstream purification strategies. To this end, Ge *et al.* showed that immunoaffinity purification followed by topdown MS holds promise as a potential clinical diagnostic approach for cTnI because it directly analyzes the entire protein at once, providing a “bird’s eye view” of its various PTMs, sequence modifications, and proteolytically degraded forms [64]. The ability of top-down proteomics to simultaneously detect multiple cTnI proteoforms is a fundamental strength of intact analysis, but obtaining sufficient sensitivity for low-abundant cTnI proteoforms in the complex matrix of patient serum remains a challenge compared with a well-designed targeted MRM-MS or ELISA based methods. Furthermore, the de-convoluting the charge envelope of larger proteins in top-down MS is a technically difficult but rapidly evolving technology. Nevertheless, given the improvement of the past decade in instrument sensitivity and automation, MS-based PTM analysis of cTnI is approaching the clinically used ELISA-based diagnostic assays.

The best-approach to establishing a clinically relevant diagnostic assay for modified proteoforms of cTnI requires the use of an automated immunoassay with multiple monoclonal antibodies that not only capture cTnI, but also its many phosphorylated, acetylated, O-GlcNAcylated, and otherwise modified variants. A topdown MS-approach could then be employed to provide a measure of relative abundances of modified variants. Alternatively, a bottom-up targeted approach involving a well-designed and validated MRM

assay could provide quantitative information relating to specifically modified residues. Currently, the comprehensive analysis of cTnI variants under these approaches requires multiple assays and multiple preparation protocols. Our laboratory has developed a quantitative cTnI assay using one capture antibody and multiple detection antibodies with distinct epitopes, thus quantifying 5 different epitopes simultaneously in a single sequential assay [18]. This approach can be adapted to the determination and quantification of cTnI along with its degradation and phosphorylated variants, as well as cTnI bound in its troponin complex. If applied specifically to the detection of cTnI phosphorylation on multiple sites, the multiple detection antibody approach could be the only assay capable of achieving sufficient sensitivity for the direct analysis of phosphorylated cTnI variants in patient blood. This analysis would represent a valuable tool in applying phosphorylated cTnI variants to the diagnosis of heart disease, disease progression, and risk group stratification.

8. Conclusion

Cardiac Troponins are the most commonly used clinical biomarkers for the diagnosis of acute myocardial infarction and for risk-stratification of acute coronary syndromes. The troponin assays that form the analytical basis for its clinical utility have undergone dramatic improvements over the past decade, and are currently capable of quantitatively detecting baseline levels of low-abundant circulating cTnI in the absence of MI. These technological advancements have provided clinicians and researchers with an opportunity to not only address important questions surrounding circulating cTnI in the improved diagnosis of specific patient cohorts, but also to apply its quantitative analysis in predictive and prescriptive ways that directly address the need for more personalized medical care.

Additionally, the advent of high-sensitivity cTnI platforms coincides with steady improvements in MS instrumentation, and this has shed a new light on the potential value of modified cTnI proteoforms for increased diagnostic precision. Cardiac function is fine-tuned by a diversity of cellular signaling cascades and modifications to specific amino acid residues. Many of these pathways are dysregulated in disease either as compensatory changes, or as maladaptive drivers of disease phenotypes. Advances in both “top down” intact protein as well as “bottom up” peptide-level MS workflows, applied to the study of cTnI at a molecular level, have uncovered an array of PTMs, degradation forms, and binding partners. Although cTnI variants evade antibody binding and detection by traditional immunoassay platforms, their collective analysis reveals a dynamic molecular fingerprint of myocardial health. The current state of MS instrumentation precludes routine quantitative analysis of cTnI variants in plasma without extensive upstream enrichment strategies. Nevertheless, increasingly sensitive MS instrumentation combined and integrated with advances in automated sample-handling workstations are making inroads towards these types of analyses. The impact is sure to extend well beyond the diagnosis of MI, with the potential to improve care for patients with a diversity of conditions and co-morbidities, spanning from the risk for heart transplant rejection, to heart failure and hypertrophic cardiomyopathy, to the dosing of potentially cardiotoxic chemotherapeutic agents, and beyond.

9. Expert Commentary

Immunoassay-based clinical platforms that quantitatively measure circulating cTnI have undergone a revolution in terms of sensitivity to where cTnI can be routinely measured in a majority of plasma samples. This new capability endows these assays with the power to improve the diagnostic accuracy for patient cohorts that have previously been underserved. Already, high sensitivity cTnI platforms are addressing important questions to characterize circulating cTnI levels among patients with significant co-morbidities such as chronic kidney disease, and, importantly, among women. Beyond the questions of defining normal from pathological circulating cTnI values, high sensitivity platforms can provide a longitudinal quantitative profile to reveal dynamic changes at the level of individual patients. This type of cTnI analysis may find increasing clinical value in its prospective ability to identify risks for cardiovascular events as opposed to consequences thereof. These platforms are already well positioned to contribute in an immediate way to personalized and precise cardiovascular care.

Regardless of their impressive increases in sensitivity, immunoassays are inherently dependent on antibody specificity and selectivity. As such, these platforms will ultimately remain incapable of identifying or distinguishing between the array of modified circulating cTnI proteoforms. In this area, modern MS-based approaches are well suited to proteoform analysis. Several of these proteoforms are already known to reflect pathophysiological processes, as well as revealing a deeper understanding of cardiovascular function. Phosphorylation is the best characterized and most commonly studied of these modifications, but this is largely a consequence of the relative ease with which it can be detected. Many phosphorylation events can be detected by western blot analysis. Although the specificity of these antibodies may imperfect, the charge difference that phosphorylation imparts on the amino acids it modifies lends itself well to MS analysis. Conversely, O-GlcNAcylation represents a similarly dynamic modification that may compete with phosphorylation for similar residues, or act independently. Owing to its relatively recent discovery, because it does not impart any charge difference, and because relatively few antibodies target specific O-GlcNAcylated proteins, this modification is much less understood. This is similarly true for Citrullination, Acetylation, Snitrosylation and other PTMs. Finally, all of these modifications are stoichiometrically disfavored and require highly technical and specialized enrichment strategies upstream of MS analysis. Advances in MS instrumentation and increasingly automated sample handling workflows are steadily closing the gap between what can be achieved in research labs and what is suitable for clinical diagnosis. MS techniques can simultaneously yield targeted quantitative data as well as a snapshot of protein modifications. Despite this promise, current MS-based assays continue to lack the required sensitivity to quantify cTnI and PTMs from plasma in the absence of extensive enrichment. Furthermore, MS assays must overcome the wide dynamic range and relatively high salt content inherent to plasma. The routine adoption of cTnI PTMs as clinical biomarkers rests on the merger of robust high-sensitivity analytical instruments with automated sample preparation platforms that both decrease sample complexity to lessen the dynamic range and provide a cleanup step to render plasma-based

samples into MS-compatible. Each of these advances will combine to increase precision and throughput.

10. Five-Year View

The current state of MS instrumentation precludes routine quantitative analysis of cTnI variants in plasma without extensive upstream enrichment strategies. Nevertheless, increasingly sensitive MS instrumentation combined and integrated with advances in automated sample-handling workstations are making inroads towards these types of analyses. Within the next five years, platforms will increasingly integrate automated preparative steps with LC-MS/MS instrumentation, rendering MS-derived proteomic data both accessible and commonplace in modern clinical chemistry laboratories. The impact is sure to extend well beyond the diagnosis of MI, with the potential to improve care for patients with a diversity of conditions and co-morbidities, spanning from the risk for heart transplant rejection, to heart failure and hypertrophic cardiomyopathy, to the dosing of potentially cardiotoxic chemotherapeutic agents, and beyond.

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Abbreviations:

A	alanine
AMI	acute myocardial infarct
AMPK 5’5	AMP-activated protein kinase
Ca^{2+}	Calcium
Cl	
cTnI	cardiac Troponin I
cTnT	cardiac Troponin T
cTnC	cardiac Troponin C
ELISA	enzyme-linked immunosorbent assay
ESC	The European Society of Cardiology
HCM	hypertrophic cardiomyopathy
HF	heart failure
K	lysine
LC-MS	liquid chromatography-mass spectrometry

MI	myocardial infarction
MRM/MS	Multiple Reaction Monitoring/Mass Spectrometry
MS	mass spectrometry
O-GlcNAcylated	Glycosylation with O -linked β- N –acetylglucosamine
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PKG	protein kinase G
PTMs	post-translational modifications
QC	quality control
S	serine
SISCAPA	Stable Isotope Standards and Capture by Anti-Peptide Antibodies

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Key Issues

- Cardiac troponin I is a key molecular switch that regulates myocardial contraction
- Circulating cardiac troponin I is the gold standard diagnostic biomarker for the diagnosis of acute MI, but it is also released at low levels with normal cell turnover and with minor injuries associated with other disease states.
- Cardiac troponin I is heavily post-translationally modified in physiological and pathological conditions. Several of its modified residues have been extensively studied but a role for a majority of these modifications is yet to be defined.
- Advances in analytical technologies and instrumentation can already detect modified amino acid residues within cardiac troponin I. These platforms are continuing their evolution towards increasing quantitative accuracy, throughput, and workflow automation.
- The goal of quantitative analysis specific disease-induced modified forms of cardiac troponin I will provide additional insight into a person's risk in developing future cardiovascular insults and or heart failure.

Table 1:

Listed are the numerous identified PTMs for cTnI. For most their function or if they are linked to cardiac diseases is still unknown. PTM localizations obtained from animal studies are listed based on their orthologous locations within the human cTnI sequence.

AA	PTM	Domain	targeting enzyme	Information	Disease	Ref.
2	N-acetylalanine					[123,124]
5	Serine-O-linked b- N-acetyl-D-glucosamine (O-GlcNAc)	Acidic region of N-terminal extension	O-GlcNAc Transferase		Potentially associated with Diabetes	[125]
5	Phosphoserine	Acidic region of N-terminal extension			HF.	[19]
6	Phosphoserine				HF.	[19]
6	O-GlcNAc	Acidic region of N-terminal extension	O-GlcNAc Transferase		Potentially associated with Diabetes	[125]
23	Phosphoserine	N-terminal extension	PKA [126], PKC [127], PKD [128], PKG [129], AMPK [120], PP1 [130], PP2A[68,69]	decreases Ca2+ affinity of TnC, reduces Ca2+ sensitivity, increases relaxation velocity & contractile power [5,126,127,131,132]	HCM [68,69]. AMPK activity is inhibited in humans with progressed HF [99].	[19,68,69, 99,120, 124,126, 127,128, 129,130, 133]
24	Phosphoserine		PKA [126], PKC [127], PKD [128], PKG [129], AMPK [120]			
26	Phosphotyrosine	N-terminal extension			HF.	[19]
31	Phosphothreonine		TK4/MST1			[134]
36	Acetyllysine					[78]
42/44*	O-GlcNAc	IT arm	O-GlcNAc Transferase		Potentially associated with Diabetes	[125]
42	Phosphoserine	IT arm	PKC/PRKCE	Increases myofilament Ca2+ sensitivity; slows kinetics & stabilizes inhibition of thin filament activation [107]; in vivo pseudo-phosphorylation leads to decreased contractility & relaxation [135]	HF. Phosphorylation contributes to cardiac dysfunction.	[19,103– 105]
44	Phosphoserine		PKC/ PRKCE			[19,103– 105]
51	Phosphothreonine	IT arm	STK4/ MST1		HF.	[19,134]
76	O-GlcNAc	IT arm region	O-GlcNAc Transferase		Potentially associated with Diabetes	[125]
77	Phosphoserine	IT arm region			HF.	[19]
78	Phosphothreonine				HF.	[19]
106	Acetyllysine					[78]
117	Acetyllysine					[78]
129	Phosphothreonine		STK4/MST1			[134]
131	Acetyllysine					[78]
143	Phosphothreonine	Inhibitory region	PKC/STK4/MST1		HF.	[19,134]

AA	PTM	Domain	targeting enzyme	Information	Disease	Ref.
150	Phosphoserine		PAK3 [136]/AMPK [85,119,120]	Phosphorylation increases myofilament Ca^{2+} sensitivity [85,119,120]. Blockade of AMPK mediated phosphorylation reduces cell shortening & electrical conduction abnormalities [121].	AMPK activity is inhibited in humans with progressed HF [99].	[85,99, 119–121, 136]
150	O-GlcNAc		O-GlcNAc Transferase	Potentially decreasing Ca^{2+} sensitivity	HF	[74]
166	Phosphoserine	Switch peptide between 2 actin binding regions	PKA [137]		HCM [68,69], HF	[19]
167	O-GlcNAc	Switch peptide between 2 actin binding regions	O-GlcNAc Transferase		Potentially associated with Diabetes	[125]
174	Acetyllysine					[78]
177	Acetyllysine					[78]
181	Phosphothreonine	C-terminal region			HF	[19]
193	Acetyllysine					[78]
199	O-GlcNAc	C-terminal region	PKCα [138]	Pseudo-phosphorylation increases myofilament Ca^{2+} sensitivity for force development most likely by reduced actin & actin-TM binding [118].	Increased phosphorylation in end stage HF [19]. Mutation found in HCM & is associated with arrhythmias & sudden death [139].	[19,118, 138,139]
	O-GlcNAc	Potentially cTnI	O-GlcNAc Transferase	Compensatory/ adaptive in HF	O-GlcNAc is critical in HF; Hypertrophy	[75,73]

*Asterisk denotes ambiguity in PTM site mapping.