Modified Troponin I as a Candidate Marker for Chronic Heart Failure
A Top-Down Perspective

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Study Hypothesis
Despite the high incidence and mortality associated with chronic heart failure (CHF), few biomarkers for its diagnosis are approved for clinical practice. Therefore, new markers and diagnostic options are the object of intense research. In this study, Zhang et al address this need by testing their hypothesis that the levels of modified cardiac troponin I (cTnI) correlate with the development of HF. Intriguingly, quantification of cTnI is well established in clinical practice for the assessment and diagnosis of myocardial infarction. Consequently, the team precisely quantified 2 well-established post-translational modifications (PTMs) of cTnI (ie, phosphorylation and truncation) in tissue specimens from healthy and gradations of diseased hearts. The study eloquently demonstrated a correlation between the levels of phosphorylated cTnI and the degree of deterioration in cardiac function.

How Was the Hypothesis Tested?
Modified forms of cTnI were assessed in the study. Notably, PTMs are emerging as new determinants of phenotypic complexity, potentially making them more descriptive of a specific biological condition, including disease, than aggregate protein levels. In fact, the fast-paced development of technological platforms that separate, measure, and characterize several thousands of proteins at once (ie, proteomics) has revealed that PTMs occur surprisingly frequently, no less in the heart.1

To study entire proteomes (ie, the protein constitution of a biological system, including sequence variants and modified forms), two approaches have been developed. Historically, proteins have been characterized using mass spectrometry (MS) after they are digested into peptides, an approach known as bottom-up or peptide-centric proteomics. This is because of the improved solubility of peptides with respect to proteins, which allows a better recovery. Also, the fact that the error associated with the measurement of a molecule’s size in MS is proportional to the size itself has contributed to the success of the bottom-up approach. However, in recent years, the introduction of MS with high resolution and accuracy, such as that offered by Fourier-transform (FT) instruments used in this study, has permitted researchers to assess proteins in their full complexity, before digestion. The second approach, known as top-down or protein-centric proteomics, was used in the study by Zhang et al.

As the authors describe, the top-down approach offers the advantage of resolving differently modified forms of the protein in an equitable fashion and obtaining quantitative measurements, provided that the level of each analyte surpasses the sensitivity threshold of the method. Zhang et al used postmortem samples (stored at 4°C for up to 34 hours) to increase the number of observations, but the main findings were validated using “fresh” snap-frozen samples. In fact, sample collection is a critical step in proteomic studies because PTMs are readily altered by changes in the environment.

In all, cTnI was extracted from 36 clinical tissue samples. These included 22 postmortem heart tissues from 7 normal patients, 5 with early-/mild-stage hypertrophy, 4 with severe hypertrophy/dilation, and 6 with overt CHF. In addition, 16 fresh “transplant” samples were analyzed, including 4 healthy donors and 10 end-stage failing hearts (ischemic/dilated cardiomyopathy, or ICM/DCM). Troponin complexes were purified using affinity chromatography with 2 different immobilized antibodies for the N- and C-termini (antibodies 14G5 and MF4, respectively). After elution, cTnI complexes were separated and desalted using reverse-phase chromatography and analyzed in an FT ion cyclotron resonance MS. Protein fragmentation, which is needed to “sequence” the protein and assign the exact site of modification to a specific residue, was obtained in the MS with either collision-induced dissociation or electron-capture dissociation. More important, the latter method allowed for a “catalyzed” fragmentation, which prevents PTMs from being lost while the protein backbone is being fragmented.

Principal Findings
The FT ion cyclotron resonance analysis resolved and quantified the monophosphorylated and biphosphorylated forms (p and pp, respectively) of cTnI and a few differentially
C-terminal–truncated forms of N-terminally acetylated cTnI. Other major and minor degradation products included internal degradation products. Twenty-two different forms of cTnI were detected in postmortem samples, including monophosphorylated, biphosphorylated, and several degraded forms, whereas the same phosphorylated forms, but only 2 degradation products, were detected in the fresh samples. The only phosphorylation sites detected in the study were at the Ser22 and Ser23 residues, with only Ser22 being phosphorylated in both the monophosphorylated and biphosphorylated peptides. This indicates that Ser22 and Ser23 are the principal phosphorylation sites in both healthy and failing hearts in vivo, occurring at frequencies exceeding the detection threshold of the method (≥1% of the total cTnI population).

For postmortem samples, a clear trend was detected, with total phosphorylated cTnI significantly decreasing, along with the diminished cardiac function in postmortem tissues (normal > hypertrophy > severe hypertrophy/dilation > CHF). This was a reflection of the stable decrease of both p-cTnI (from 34.2±2.1% to 0.7±0.4%) and pp-cTnI (from 18.4±2.4% to 0.3±0.2%) from normal to CHF samples. These findings were confirmed in fresh samples, in which both p-cTnI and pp-cTnI were markedly decreased in ICM/DCM compared with healthy donors (38.6±3.8% to 16.7±2.4% and 10.9±2.2% to 2.2±0.6%, respectively).

Although a striking correlation between the abundance of phosphorylated cTnI and cardiac functional status was observed, protein degradation did not appear to be associated with function. In fact, the analysis of several truncated forms of cTnI did not show significant changes among different pathological states. Also, no correlation was found between protein degradation and phosphorylation state.

**Implications**

This study demonstrates a sustainable workflow for the discovery of clinically useful biomarkers through a proteomic pipeline. At least two features of the experimental design are notable. First, the study is conducted using heart tissue rather than blood samples. Discovery of biomarkers (normally low-abundance proteins) directly in plasma is complicated by the wide dynamic range of its proteome and the presence of abundant proteins (eg, albumin), which “cloud” less abundant proteins. Second, the authors detected and measured multiply modified forms of a protein, in this case cTnI. This raises the prospect of follow-up studies addressing other highly abundant cardiac proteins (eg, sarcomeric proteins) and their modifications. This could potentially lead to the development of panels of biomarkers that would better discriminate between normal and pathological states than any single biomarker. Because data collection proceeds in an unbiased fashion and data from a wide variety of healthy and diseased clinical specimens are accumulated, the knowledge generated by targeted proteomic studies will represent a valuable patrimony for future diagnostic tests.

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**Disclosures**

None.

**Reference**

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