the sum of UIBC and Fe concentrations theoretically yields TIBC. Recent work by Gambino et al. [11] demonstrated that when Fe and UIBC (summed for TIBC) and transferrin were measured precisely and without bias, the ratio of TIBC to transferrin (g/L) is close to the theoretically expected value of 25.0.

Further standardization activities for these analytes may well be required, as it was for cholesterol, if acceptable accuracy and precision are to be achieved. Clearly, additional reference materials and expanded proficiency testing programs are highly desirable. We believe, however, that the TS assay, with Fe/TIBC or Fe/UIBC, is a viable screening test for hemochromatosis and could be widely used as part of routine physical examinations to detect this condition, which is frequently not diagnosed until its serious sequela such as cirrhosis, arthritis, and cardiomyopathy occur.

References
3. Kahn J. Serum iron levels: is more or less iron better? Clin Lab News 1997;23:1,2.

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Letters

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Markers of Myocardial Damage

To the Editor:

We read with interest the editorial by Collinson on the use of cardiac troponins T and I (cTnT, cTnI) as specific markers of myocardial damage [1]. We agree with Collinson when he suggests that there were some inconsistencies in the data presented by Bodor et al. [2]. We, too, were concerned by the use of a system optimized for the measurement of serum troponin T to measure this analyte in extracts of muscle tissue, since there are several dilution and matrix problems to address in this setting [3]. Bodor et al. have previously published data measuring cTnI, using the same extraction method [4]. We would suggest that, if the methods were as described in both of these publications, they appear to be fundamentally flawed. The authors omitted a vital step in the purification process, which renders their data highly questionable.

They prepared two separate fractions, one for soluble cytosolic proteins and one for insoluble myofibrillar proteins. The myofibrillar fraction was then solubilized with 8 mol/L urea. The detergent action of urea denatures the proteins of the myofibrillar complex, causing the components of the complex to dissociate. It is well documented that even low concentrations of urea interfere with the Lowry protein assay [5, 6]. The kit manufacturer, Sigma, does not recommend the use of this assay in the presence of urea. However, Bodor et al. used this assay to determine total protein concentration in the presence of 8 mol/L urea. The consequent problem is illustrated in Fig. 1. In addition, 8 mol/L urea could seriously interfere with antibody binding in the immunoassay for cTnT and contribute to a substantial matrix effect. The myofibrillar fraction should have been desalted before any analytical measurements for either total protein, cTnI, or cTnT were performed. Several studies have been published in which urea was used in the protein purification process. However, urea was removed before analysis [7–9].

We have repeated the method of Bodor et al. with the addition of a desalting step. We measured cTnI (Sanoﬁ) and cTnT (Boehringer Mannheim, second-generation assay) in samples from adult human heart and skeletal muscle. The cardiac muscle contained 122 and 59.0

![Absorbance vs Urea](image)

Fig. 1. Absorbance at 600 nm observed with the Lowry method for the measurement of total protein (Sigma kit), in the absence of any protein and increasing concentrations of urea.
mg/g protein of cTnI and cTnT, respectively. In skeletal muscle the comparable results were 0.02 and 0.001 mg/g protein, respectively. The skeletal muscle results represent <0.2% and <0.01% of the cardiac concentrations for cTnI and cTnT, respectively, and are consistent with previously reported data [10, 11].

Bodor et al. used three techniques: immunohistochemistry, Western blot, and immunoassay of tissue extracts. They presented impressive Western blot data. However, these contradict their data from ELISA measurements in tissue extracts, and also their discussion. The only data that actually supported their discussion was the immunohistochemistry, but it is worth noting that immunohistochemistry is, at best, semiquantitative, and a polyclonal antibody was used for this technique. In Bodor et al.’s earlier paper [4] they attempted to measure cTnI in heart and skeletal muscle. They described in depth the method of analysis in the methods section, but in the results section gave no results for any of their analyses in cardiac tissue. Why?

From the data of Bodor et al. it would seem reasonable to suggest that, taking the two papers together, the issue of whether cTnT and cTnI are present in muscle tissue other than of cardiac origin is far from resolved.

References

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Three of the authors of the paper referred to reply.

To the Editor:
We thank Fredericks et al. for their interest in our recent article [1] and for their comments regarding this same publication. We recognize that issues regarding cardiac troponin T (cTnT) expression are unresolved. However, we disagree with Fredericks et al. on whether cTnT is present in skeletal muscle. Our data prove that cTnT is present in some skeletal muscle. The unresolved issue is: What is the isoform of cTnT expressed in regenerating skeletal muscle? Is it the adult cardiac or the fetal cardiac isoform? The answer to this question must come from additional studies.

Fredericks et al. ask several questions in their Letter. It appears that most of these questions originate from misunderstanding our data. Their main concerns are clustered around the following four issues: (a) possible matrix effect while measuring cTnT in tissue extracts with an ELISA optimized for serum measurement; (b) the influence of 8 mol/L urea on the cTnT ELISA and on the Lowry protein assay; (c) apparent inconsistencies in our data; and (d) what the cTnT immunoassay results were in the human heart extracts. We address these points as follows.

For the first issue, matrix effect was minimized while measuring cTnT concentration in skeletal muscle extract by using cTnT assay diluent supplied by the kit’s manufacturer. The muscle extracts were diluted in multiple steps from 10- to 500-fold in assay diluent, thus effectively minimizing or even eliminating the potential matrix effects on the cTnT ELISA. An indirect proof of lack of matrix effect can be found in Table 1 of our article [1]. Careful study of that data reveals that several skeletal muscle samples had no detectable concentrations of cTnT. This would be impossible if the detected cTnT concentration had been a false-positive reading due to matrix effect. Since all samples were prepared by the same method and contained the same buffer components, any matrix effect that produced a false-positive result would have affected all samples equally. We emphasize that sample dilutions were made such that several dilutions of the same sample would give a final cTnT concentration that would fall in the assay’s working range. The resulting concentration multiplied by the dilution factor would give the final cTnT concentration. Multiple corresponding results of any individual samples were used to ensure correct results. For the second point, use of chaotropic agents such as urea to purify regulatory proteins of muscle has been tried and found acceptable by several authors previously [2, 3]. Such purification yields troponins that are immunologically identical to the native proteins [4, 5] and they retain their physiological functionality such as ability to form complexes with troponin C [2]. As a matter of fact, a high concentration of urea (or KCl salt) is mandatory for stable storage of purified troponin I as evidenced by numerous publications, including those cited by Fredericks et al. [3, 5]. In the absence of urea, KCl, NaCl, or troponin C, purified troponin I will be lost from solution because of adherence to the surface of the containing vessel (unpublished data).

To the Editor:

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A high urea concentration causing a diminished antigen–antibody reaction in the cTnT ELISA would have caused decreased or even undetectable cTnT concentrations instead of the positive reading. By extension of the argument of Fredericks et al., if one assumes a diminished antigen–antibody reaction, one must conclude that skeletal muscle contains even more cTnT than what we demonstrated in our publication.

It is true that high urea concentration will increase absorbance reading in the Lowry assay in the absence of any protein, but the effect of this on the calculated protein concentration can be reduced or eliminated. In our experiments we minimized the effect of urea by three different means. One method was to use a 100-fold dilution of the muscle extract in water. This dilution effectively lowers the urea concentration in the sample to 0.08 mol/L, and according to Fig. 1 cited by Fredericks et al., this urea concentration has no effect on the Lowry assay. The second method of eliminating urea interference was to use appropriately prepared blanks that contained urea concentrations similar to that of the sample. Thus any nonspecific increase of absorbance was canceled out by “blanking” the assay with this preparation. The third method was to use protein precipitation, as suggested by the Sigma product literature, to verify our protein assay results by eliminating chemical interferences in the Lowry assay. We point out that each Lowry assay. The second method of conducting by several people at multiple locations, independently of each other and without knowledge of the other laboratories’ results.

On the third issue, we are very glad that Fredericks et al. pointed out the apparent inconsistencies in our data. We believe that their interpretation of our data is incorrect and therefore we welcome the chance to provide an explanation. We use panel D of Fig. 4 [1] as our example. The histochemistry slide presented on this panel was cut to uniform thickness; therefore we can use the area presented on the panel for our calculation. Simple measurement of dimensions of the presented photograph reveals that the total area of panel D is 1855 mm² (53 × 35 mm). The area of the single muscle fiber expressing cTnT is ~1.5 mm². Assuming that all the troponin T in the single degenerative fiber is cTnT, we can conclude, on the basis of the area measurement, that <0.08% of all troponin T in the depicted tissue section is cTnT. However, the photomicrograph represents only about 1% of the whole cross section of the biopsy specimen, and the remaining skeletal muscle tissue of this specimen did not contain cTnT either. Therefore one can conclude that in this skeletal muscle sample, <0.0008% of the troponin T is cTnT.

The result from the above calculation can be applied to explain the findings of the Western blot [1]. Lane 3 contains extract from one skeletal muscle sample containing 50 μg of total protein, only part of which is troponin T. This allows for not more than 0.0004 μg (or 0.4 ng) of cTnT to be loaded on lane 3. This amount is ~10-fold below the detection limit of our Western blot assay. It is, therefore, not surprising that no cTnT could be seen in lane 3 of Fig. 5 [1]. However, the increased cTnT concentration in DMD skeletal muscle could be detected even by the Western blot assay (lane 4), in perfect agreement with the immunohistochemical and ELISA assay results [1]. One of the reasons for utilizing immunohistochemistry to detect cTnT in normal and diseased skeletal muscle was the above-demonstrated lower detection limit of this technique. With immunohistochemistry, one can process an arbitrarily large section of tissue and detect the presence of the chosen antigen even if it is present in only a very minuscule number of the cells in the tissue. This is obviously impossible if some other extraction and detection methods are used.

On the basis of the above-presented data, we can conclude that negative Western blot, positive ELISA, and positive immunohistochemistry for cTnT in normal skeletal muscle is not the sign of inconsistency in our results but a phenomenon explained by the different detection limits of the various analytical methods.

As we indicated in our Discussion [1], we sought to find a possible physiological explanation for the well-documented positive cTnT ELISA results in several groups of patients who do not have any clinical evidence of cardiac muscle injury. We reserve judgment on the clinical significance of the presence of cTnT in <0.0008% of fibers of normal skeletal muscle. However, the theoretical significance of our findings, as we stated in the Discussion, is that cTnT can be present in apparently normal skeletal muscle and it is almost always present in diseased skeletal muscle tissue. Although the chances are minor that the relatively small amount of cTnT present in skeletal muscle could be released into the circulation to give an increased concentration in serum, it is still a potential clinical consideration for an increased cTnT result that is a false positive for myocardial injury.

Addressing the last issue, the human heart extracts contained the following amounts of cTnT (mean ± SD): right ventricle, 13.88 ± 1.46 mg cTnT per g wet tissue; left ventricle, 10.59 ± 2.69 mg/g. We chose not to include these results in our paper for two reasons. One, the heart tissue was used as a control in our experiments. Our interest was to assess the presence or absence of cTnT in human skeletal muscle (as the title of our paper clearly indicated). Second, the results of these experiments had already been published [7].

Finally, we express our concerns about Fredericks et al.’s data. If they really desalted the muscle extracts before performing the cTnI or cTnT assays, they could have lost a significant portion of the myofibrillar proteins, especially cardiac troponins I and T; thus the presented quantification data could be flawed. Unfortunately, they did not provide any details about their experiments and their “desalting” method. If they fol-
lowed the methods of the references they cited in their letter, then the 8 mol/L urea buffer was replaced with buffer containing either 1 mol/L KCl or 0.5 mol/L NaCl. In either case the effect of the high salt concentration on the Lowry assay is not negligible. For example, the reference cited by Fredericks et al. [6] states that the maximum “tolerable limit” of KCl is 12 mmol/L. We would be extremely interested in how Fredericks et al. can explain their results in light of this information. Similarly, the presence of 1 mol/L KCl in the sample questions the validity of Fredericks et al.’s cTnT immunoassay results. The disruptive effect of the high salt concentration on the antigen–antibody reaction has been well documented and exploited, for example during immunoaffinity chromatography. Therefore, negative results of the cTnT ELISA for samples with 1 mol/L KCl or 0.5 mol/L NaCl cannot be accepted as proof of absence of cTnT in the sample.

References