Fluoroenzymometric method to measure cardiac troponin I in sera of patients with myocardial infarction

Martina Zaninotto, Sara Altinier, Mattia Lachin, Paolo Carraro, and Mario Plebani

The aim of our study was to evaluate the clinical relevance of serum troponin I (TnI) as a marker of ischemic myocardial injury by using an automated fluoroenzymometric assay. The reference range for serum TnI was established by measuring serum TnI concentrations in blood from 75 healthy donors. The concentration was then compared with serum creatine kinase (CK) activity, CK-MB mass, and myoglobin concentrations in 20 patients with myocardial infarction diagnosed according to the WHO criteria, 20 patients with chest pain of nonischemic origin, 9 patients with unstable angina, 11 with stable angina, 11 patients with chronic muscular diseases, 6 patients with muscular trauma without chest contusion, and 13 patients with chronic renal disease. We found that: 

(a) 99% of the blood donors had TnI concentrations <0.26 μg/L (detection limit of the assay in our study); 
(b) TnI values in acute myocardial infarction (AMI) patients 4 h after onset of chest pain showed a sensitivity of 0.769 and a specificity of 1.0 at a decisional concentration for AMI of 1 μg/L, even in the presence of severe skeletal muscle injuries or renal diseases; 
(c) the increase in TnI concentrations after infarction (interquartile range 3.25–6 h) and the peak occurred later (interquartile range 11.5–24 h) than the rise found in myoglobin and CK-MB, but the increase persisted much longer (>96 h); 
(d) receiver-operating characteristic curve analysis showed the high diagnostic accuracy of TnI in diagnosing AMI even in patients in whom traditional biochemical markers are adversely influenced by underlying clinical situations.

INDEXING TERMS: creatine kinase • creatine kinase MB • myoglobin • diagnostic efficiency • biological markers

It is widely accepted that the sensitivity and specificity of serum enzymes and isoenzymes such as total creatine kinase (CK; EC 2.7.3.2), CK-MB activity, and lactate dehydrogenase, the final arbiters by which myocardial damage is diagnosed or excluded up to now, are inadequate [1, 2]. Several new biochemical markers therefore have been investigated recently for the early and accurate diagnosis of acute myocardial infarction (AMI) [3–6]. Because the contractile and regulatory proteins of the myocardium [7, 8] have high concentrations in myocytes and are expressed as tissue-specific isoforms, they appear to be potentially useful innovative biological markers for sensitive and specific diagnostic tests [9–11].

In particular, troponin I (TnI), the inhibitory protein of the troponin–tropomyosin complex, has three isoforms, two skeletal and one cardiac (sTnI and cTnI respectively), that are encoded by three distinct genes, presenting a dissimilarity of ~40% in the amino acid sequence [12]. Moreover, human cardiac TnI has 31 additional residues on its N-terminal end that are not present in skeletal forms, thus providing a high potential for obtaining cardiac-specific antibodies [13, 14]. Furthermore, since the molecular mass of human cardiac TnI is 24 kDa, the protein is released fairly rapidly after AMI. This property and the unique amino acid sequence of cardiac TnI make it a promising candidate as a diagnostic marker for myocardial damage [15, 16].

The aims of our study were therefore to evaluate the analytical performance of a fluoroenzymometric method previously proposed for cardiac TnI measurement [17] and compare its diagnostic accuracy with that of traditional markers of myocardial injury in the diagnosis and monitoring of patients with AMI.

Materials and Methods

The study was carried out on 165 patients subdivided as follows:

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3 Nonstandard abbreviations: CK, creatine kinase; AMI, acute myocardial infarction; and TnI (TnT), troponin I (troponin T).
Group 1. Seventy-five healthy subjects (36 men and 39 women), ages 18 to 73 years (median 45 years), with no history of cardiovascular disease, as controls to obtain a reference interval.

Group 2. Twenty patients (13 men and 7 women, ages 46 to 87 years, median 67 years) with AMI diagnosed according to the WHO criteria (characteristic chest pain, electrocardiographic findings, serial increases of total CK activity and CK-MB concentrations with peak values of twice the upper limit of the reference interval): 16 with Q-wave and 4 with non-Q-wave infarctions admitted to the coronary care unit within 9 h after the onset of symptoms (range 0.5–9 h; median 3.5 h); 14 given a thrombolytic treatment while 6 were not treated, depending on clinical circumstances, and 12 of these classified as reperfused on the basis of clinical pictures (symptoms resolved rapidly, ST-segment changes improved, presence of reperfusion arrhythmias) [18].

Group 3. Forty patients, 20 with chest pain of nonischemic origin, 9 with unstable angina (five patients assigned to class III, two patient to class II, and two to class I according to Braunwald’s classification [19]), and 11 with stable angina, to evaluate the usefulness of new marker for ruling out AMI in emergency department patients.

Group 4. Patients with chronic skeletal muscle diseases (11), multiple traumas without chest contusion (6), and with chronic renal failure (13), to compare the clinical specificities of the tests in a worst-case situation. In these patients the concomitant myocardial injury was excluded by the usual criteria, i.e., no history of chest pain and no electrocardiographic signs of ischemia within the previous 3 weeks.

BLOOD SAMPLING
Only one blood sample was drawn from groups 1 and 4 patients. Blood samples were collected from AMI patients (group 2) immediately upon admission to the coronary care unit, and then hourly for the next 6 h, every 3 h until 24 h, and then every 6 h until the fourth day after admission. In patients with unstable angina (group 3), the blood samples were collected at 4-h intervals during the first 24 h and at 6-h intervals until 2 days after hospitalization, whereas in patients with chest pain and stable angina (group 3), one blood sample only was drawn. All patients gave their informed consent for extra blood samples to be drawn.

In serum samples obtained from group 1 patients, TnI only was assayed, whereas in each serum sample from groups 2, 3, and 4 patients, TnI, total CK activity, CK-MB (mass concentration), and myoglobin were measured.

TnI
TnI concentrations were assayed by a commercially available automated two-site immunoassay (Stratus Cardiac Troponin I; Baxter Dade, Milano, Italy) [20] that includes two monoclonal antibodies specific for cardiac isotype of the protein. The measuring range of the method was from 0.35 (analytical sensitivity declared by the manufacturer) to 50 μg/L, the assay generating TnI results within 10 min.

Three control samples containing different concentrations of TnI (Baxter Dade Stratus Troponin I controls) and a pool of patients’ sera were used in each run to verify the imprecision of the method, whereas the detection limit of the assay (mean absorbance + 3 SD of the zero calibrator) was verified by running 10 replicates of the zero calibrator in different runs.

TOTAL CK ACTIVITY
The catalytic concentration of CK was determined according to the method recommended by the IFCC [21] at 37 °C (Bracco, Milano, Italy). The decisional concentrations for AMI used in this study were >160 U/L for women and >190 U/L for men.

CK-MB
The mass concentration of CK-MB was measured with a commercially available immunoabsorbant assay (Stratus CK-MB; Baxter Dade) with a sensitivity of 0.4 μg/L. The discriminator value for AMI was 5 μg/L [22] and 2.5 for the relative index [CK-MB (μg/L)/total CK (U/L)] × 100.

MYOGLOBIN
The serum myoglobin concentration was assayed with the immunological latex method (Istituto Behring; Scoppito, L’Aquila, Italy), and a discriminator value for AMI of 50 μg/L was chosen [23].

STATISTICAL EVALUATION
To compare the results from cases in which the time elapsed between onset of chest pain and admission to the coronary care unit was different, we added this individual time to that of each blood sampling and produced 24 time series of 1 (until 6 h), 3 (until 30 h), and 5 (until 4 days) h. Each result was thus included in the appropriate time series.

Medians and percentiles were calculated to describe continuous variables.

To evaluate the efficiency of the markers, we used a PC program called “Test Evaluation” supplied by W. Gerhardt (Department of Clinical Chemistry and Section of Cardiology, Lasaretet, Helsingborg, Sweden) and applied it to an IBM computer [24]. The test produces comparative graphs by making double histograms with overlapped sensitivity, specificity, and efficiency curves. Then, by plotting the sensitivity vs 1 – specificity, receiver-operating characteristic (ROC) curves were constructed to compare the discriminative power of the methods tested. The diagnostic efficiency was ascertained on the basis of the area under the ROC curve. Areas under the curves and differences between ROC curves were calculated according to the method proposed by Hanley and McNeil [25, 26], with the Graph ROC for windows program by Veli Kairisto and Allan Pooa (Department of Clinical Chemistry, University of Turku, Turku, Finland). Confidence intervals (95%) were calculated by Confidence Interval Analysis microcomputer program [27].
Table 1. Analytical Imprecision

<table>
<thead>
<tr>
<th></th>
<th>Assigned value</th>
<th>Within run (n = 10)</th>
<th>Between run (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( x, \mu g/L )</td>
<td>SD, ( \mu g/L )</td>
</tr>
<tr>
<td>Control sample 1</td>
<td>3.8</td>
<td>3.98</td>
<td>0.07</td>
</tr>
<tr>
<td>Control sample 2</td>
<td>16.7</td>
<td>16.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Control sample 3</td>
<td>31.6</td>
<td>30.4</td>
<td>0.86</td>
</tr>
<tr>
<td>Pool</td>
<td>1.28</td>
<td>0.05</td>
<td>3.90</td>
</tr>
</tbody>
</table>

Results

The TnI assay evaluated in our study (Table 1) has a good precision, with CVs ranging from 1.39% (\( \bar{x} = 16.5 \mu g/L \)) to 5.51% (\( \bar{x} = 1.27 \mu g/L \)); the lowest detection limit (analytical sensitivity) of the measuring range in our study was 0.26 \( \mu g/L \). Furthermore, we found that the stability of the reagents (8 h at room temperature) and of the calibration curve (at least 1 month) was satisfactory.

In healthy subjects TnI concentrations had a skewed distribution, ranging from 0 to 0.3 \( \mu g/L \); >99% of values were lower than the detection limit of the assay.

The TnI concentrations observed in all patients studied and expressed as median, 10%, and 90% are reported in Table 2. With the exception of AMI and unstable angina patients, cardiac TnI could not be detected by this assay in other patients studied. In seven patients with unstable angina (class III and class II), detectable amounts of TnI without significant variations during monitoring were observed. In our patients, no cardiac events during the hospitalization and during the monitoring were clinically diagnosed, even in the patient with the highest value of TnI (0.9 \( \mu g/L \)).

A graphic test evaluation of the TnI assay in AMI is presented in Fig. 1. In Fig. 1A, the double histogram compares the frequency distributions of TnI values observed 4 h after onset of chest pain in AMI cases (group 2, upper field) with those obtained in group 3 subjects (lower field), arranged in 10 nonequidistant classes, ranging from 0 to 30 \( \mu g/L \). The sensitivity and specificity curves are a function of the test value and reflect different distributions. On the basis of these curves, a discriminator value of 1 \( \mu g/L \) yields a sensitivity of 0.769 and a specificity of 1.0 with a diagnostic efficiency of 0.943. For evaluating the clinical specificity under worst-case situations, we replotted the TnI data of AMI cases (Fig. 1B, upper field) in

Table 2. TnI concentrations (\( \mu g/L \)) in patients studied

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>10%</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic skeletal muscle diseases</td>
<td>11</td>
<td>0.00</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>Multiple traumas without chest contusion</td>
<td>6</td>
<td>0.00</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>13</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Chest pain</td>
<td>20</td>
<td>0.00</td>
<td>0.00</td>
<td>0.20</td>
</tr>
<tr>
<td>Unstable angina*</td>
<td>9</td>
<td>0.00</td>
<td>0.40</td>
<td>0.78</td>
</tr>
<tr>
<td>Stable angina</td>
<td>11</td>
<td>0.00</td>
<td>0.00</td>
<td>0.24</td>
</tr>
<tr>
<td>AMI**</td>
<td>20</td>
<td>1.00</td>
<td>2.90</td>
<td>22.88</td>
</tr>
</tbody>
</table>

*First sample available.

**4 h after onset of chest pain.

Fig. 1. Frequency distributions of TnI values in AMI patients: number of cases are plotted as a function of test values (abscissa). Sensitivity, specificity, and efficiency curves are plotted as a function of test values. On the basis of these curves, a discriminator value of 1.0 \( \mu g/L \) yielded a sensitivity of 0.769 and a specificity of 1.0 (values directly off the scale on the ordinate of the graph). (A) Upper field: frequency distribution of TnI values in AMI patients 4 h after the onset of chest pain; lower field: frequency distribution of TnI values in group 3 patients (chest pain, stable and unstable angina). (B) Upper field: frequency distribution of TnI values in AMI patients 4 h after the onset of chest pain; lower field: frequency distribution of TnI values in groups 3 and 4 (chronic skeletal muscle diseases, multiple traumas without chest contusion, chronic renal failure) patients.
comparison with those observed in groups 3 and 4 patients (Fig. 1B, lower field). No difference in the clinical specificity of the TnI assay was observed in two clinical conditions considered. The performances of all markers considered in our study were calculated in the described population with the same approach (Table 3). During the first 4 h after onset of chest pain, myoglobin showed the highest sensitivity, followed by CK-MB and TnI, whereas total CK was significantly less sensitive. On the other hand, during ~3 days of monitoring, we observed for all tests a time-dependent sensitivity (Fig. 2). In particular, during the first 2 h after the onset of symptoms, myoglobin showed the highest sensitivity (1.0), while those of total CK, CK-MB, and TnI were ~0.60. The maximum value of sensitivity (1.0) was observed from 7 to 24 h for CK-MB and from 10 to 21 h for total CK. TnI showed a constantly high sensitivity (1.0) from the 10th hour forward, while, as expected, those of the other markers progressively decreased, being 0.20 (myoglobin), 0.37 (CK-MB), and 0.62 (total CK) 70 h after the onset of symptoms. Furthermore, the clinical specificity of all markers studied, except that of TnI, decreased markedly if the evaluation was carried out in a worst-case situation, i.e., in patients with concomitant skeletal or renal diseases.

In another approach for assessing the diagnostic efficiency of TnI compared with that of the traditional biochemical AMI markers considered in our study, ROC curves were generated (Fig. 3). Despite minor differences, the area under the TnI ROC curve (0.932; 95% confidence interval 0.83–1.0) was almost the same as the unit area under the CK-MB curve (0.956; 95% confidence interval 0.87–1.0) and total CK (0.920; 95% confidence interval 0.81–1.0), showing no significant difference in comparison with the ROC curve for myoglobin (0.960; 95% confidence interval 0.88–1.0), the last being the most useful marker in diagnosing myocardial infarction within 4 h after the onset of chest pain (Fig. 3A). On the other hand, in patients with concomitant skeletal muscle damage or with renal diseases (Fig. 3B), the ROC curve for TnI (0.926; 95% confidence interval 0.82–1.0) was greater than that for CK-MB (0.830; 95% confidence interval 0.68–0.97) and significantly different from those for total CK (0.685; 95% confidence interval 0.51–0.86; z-score = 2.604) and myoglobin (0.485; 95% confidence interval 0.11–0.86; z-score = 2.236). In these patients the relative index improves the clinical specificity of CK-MB assay, the area under the ROC curve being 0.917 (95% confidence interval 0.81–1.0).

The release kinetics, and the relative increase in values of markers studied in AMI patients, are summarized in Table 4. The first increased value in TnI concentrations after infarction (interquartile range 3.25–6.0 h) and the peak (interquartile range 11.5–24.0 h) occurred later than in myoglobin and in CK-MB concentrations, but increased serum TnI concentrations persisted much longer (>96 h). Furthermore, serum TnI concentrations exceeded the discriminator limit by >158-fold.

The pattern of serum TnI release in AMI patients shows a monophasic time-dependent kinetic (Fig. 4, upper field). A marked increase in serum TnI was observed on day 1, resulting in a median peak value at ~40 times the discriminator limit at a median time of 18 h after the onset of pain. The initially released TnI was rapidly eliminated from the circulation, and a long plateau effect was observed from the second to the fourth day after onset of symptoms when TnI concentrations were persistently ~threefold the discriminator limit. Changes in TnI were similar to those of CK-MB (Fig. 4, lower field), the most striking difference being the long release of TnI, the concentrations of which remained increased >96 h after onset of chest pain. The prolonged serum increase of this marker provided a diagnostic window from 10 to at least 96 h after onset of pain, during which TnI was detectable in all serum samples of all patients. In our cases the pattern of its release in AMI patients was not influenced by reperfusion of the infarct-related artery, being monophasic in all patients.

**Discussion**

Our findings demonstrate that the fluoroenzymometric method for TnI assay has a good analytical performance. The evaluated assay, commercially available, uses the same antibodies originally developed by Bodor et al. [17], and extensively reported in experimental studies [28, 29]. The relation between the current assay and the research method calculated by the weighted least-squares equation is 0.211 (research assay)−0.019 (Ladenson JH, Division of Laboratory Medicine, Washington University, St. Louis, MO, personal communication). Thus, previously reported cutoff values cannot be taken into consideration using the commercial procedure.

We found that TnI has several advantages over traditional

| Table 3. Diagnostic performances (and 95% confidence intervals) of biochemical markers for AMI 4 h after onset of chest pain. |
|-----------------|-----------------|-----------------|-----------------|
| **Cutoff**      | **Sensitivity** | **Specificity** | **Efficiency**  |
| TnI             | 1 µg/L          | 0.769 (0.46–0.95) | 1.00 (0.95–1.00) | 0.943 (0.90–0.99) |
| CK              | 160 U/L**      | 0.692 (0.39–0.91) | 0.923 (0.75–0.99) | 0.956* (0.84–0.99) |
| CK-MB           | 5 µg/L         | 0.923 (0.64–1.00) | 0.660* (0.52–0.78) | 0.846 (0.76–0.97) |
| Myoglobin       | 50 µg/L        | 1.000 (0.75–1.00) | 0.961 (0.80–1.00) | 0.948 (0.83–0.99) |
|                 |                 |                  | 0.785* (0.66–0.88) | 0.811* (0.70–0.90) |

*Obtained by adding to group 3 group 4 patients.
**All patients (men and women).
serological markers of myocardial injury, the greatest one being its cardiac specificity [30]. TnI measurement is therefore particularly helpful in the assessment of patients with myocardial ischemia and skeletal muscle damage [31, 32]. TnI results are also easy to interpret because serum concentrations are usually very low (between 0 to 0.3 μg/L) and increase substantially (up to 158 times the discriminator) in response to an acute infarction. At the decisional concentration for AMI chosen in our study, 1 μg/L, the assay shows a good diagnostic efficiency (0.943) with an excellent clinical specificity (1.0), even in patients in whom traditional biochemical markers of AMI are adversely influenced by diverse underlying clinical situations [32, 33]. In fact, in clinical practice it is necessary to rule out AMI not only in patients with angina and other heart diseases, but also in patients with muscular or renal diseases admitted to the hospital with aspecific symptoms.

Despite its structural localization, in AMI, TnI appears in the circulation within a few hours after the onset of chest pain [34], and high concentrations persist for at least 4 days [33]. This sustained increase in the serum concentrations probably reflects a continuing release of this protein from disintegrating myofilaments; consequently there is a long diagnostic window, from 10 to at least 96 h after onset of pain, during which the TnI concentration exceeded the discriminator limit for AMI in all serum samples of our patients. This release pattern, in common with that of other contractile proteins, increases the likelihood of a positive test result, particularly in the subacute phase of infarction [8, 9].

The serum release kinetic observed for TnI in our study was monophasic in all patients studied, irrespective of the outcome of thrombolytic treatment. This behavior, different from that observed in some studies for other structural proteins such as troponin T (TnT) [9, 11], may suggest that the rapid loss of the cytoplasmic pool was superimposed on prolonged myofibrillar degradation, the content of free cytoplasmic pool of TnI being lower than that reported for TnT (~3% vs 6%, respectively) [28]. Nevertheless, this finding must be confirmed in a larger population because the results reported in the literature by different authors are contradictory [31, 35]. In a larger population, the diagnostic potential of the TnI assay for detecting minor myocardial damages should also be verified; no conclu-

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Fig. 2. Diagnostic sensitivity of TnI, total CK activity, CK-MB mass concentration, and myoglobin in relation to hours after onset of AMI symptoms.

Fig. 3. ROC curves for total CK activity, CK-MB mass concentration, TnI, and myoglobin 4 h after the onset of chest pain.

(A) Values of specificity obtained by using group 3 patients (chest pain, stable and unstable angina) as comparison group. (B) Values of specificity obtained by using together groups 3 and 4 patients (chronic skeletal muscle diseases, multiple traumas without chest contusion, chronic renal failure) as comparison group.
Table 4. Release kinetics for TnI, CK-MB (mass concentration), total CK activity, and myoglobin in myocardial infarction.

<table>
<thead>
<tr>
<th>Cutoff</th>
<th>TnI 1 µg/L</th>
<th>CK-MB 5 µg/L</th>
<th>CK 190 U/L (M)</th>
<th>Myoglobin 50 µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (h) to first increased value (50%)</td>
<td>4.50</td>
<td>4.50</td>
<td>4.50</td>
<td>3.50</td>
</tr>
<tr>
<td>(25%, 75%)</td>
<td>3.25-6.00</td>
<td>3.25-4.87</td>
<td>3.25-6.00</td>
<td>2.50-4.50</td>
</tr>
<tr>
<td>Time (h) to peak (50%)</td>
<td>18.00</td>
<td>15.00</td>
<td>20.00</td>
<td>6.00</td>
</tr>
<tr>
<td>(25%, 75%)</td>
<td>11.50-24.00</td>
<td>10.00-20.00</td>
<td>14.25-27.00</td>
<td>6.00-10.00</td>
</tr>
<tr>
<td>Time (h) of normalization (50%)</td>
<td>&gt;96</td>
<td>70</td>
<td>90</td>
<td>24</td>
</tr>
<tr>
<td>Multiples of the discriminator limit for AMI</td>
<td>158</td>
<td>110</td>
<td>37</td>
<td>53</td>
</tr>
</tbody>
</table>

The unique properties of this protein and, in particular, its high diagnostic specificity (1.0) and sensitivity in the early phase of AMI (0.769 during 0–4 h after onset of pain), the high concentration gradient between myocardial cells and normal blood, and the stability and rate of elimination suggest that the TnI measurements could become a valuable differential diagnostic tool to investigate myocardial damage in emergency room patients, even in cases of concomitant skeletal muscle injury, as occurs in polytraumatized patients. In fact, as reported in our study, the measurement of the traditional markers in these patients is inefficient due to its lack of specificity. In our experience myoglobin remains the more sensitive marker in the earlier phases of AMI. Differences in the sensitivity of this marker described in the literature [34, 36, 37] can be attributed to the lack of standardization and optimization of methods used for its measurement.

In conclusion, the determination of TnI in combination with myoglobin, the earlier marker of myocardial damage, allows a reliable and timely diagnosis to be made; myocardial necrosis to be ruled out in patients with renal failure [33] or with skeletal muscle myopathies; and the course of myocardial damage to be monitored during therapy.

We are indebted to R. Brandolese (Servizio di Anestesia e Rianimazione, Padova, Italy), C. Angelini (Clinica Neurologica, Padova, Italy), and A. Piccoli (Istituto di Medicina Clinica, Padova, Italy) for the valuable cooperation in collecting sera and data from patients.

References


