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# Cardiac specific titin N2B exon is a novel sensitive serological marker for cardiac injury

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Cardiovascular diseases are globally a major cause of mortality, with myocardial infarction (MI) presenting as the most frequent acute clinical finding. Early diagnosis is key component for the effective treatment and management of this malady. Modern diagnosis of MI relies on the electrocardiography and the measurement of serum biomarkers [1]. Measurement of cardiac troponins has been shown to be superior to all other clinically available biomarkers, such as myoglobin, the MB-fraction of creatine kinase (CK-MB), for the diagnosis of myocardial infarction. However, the cardiac troponin assays have their limitations. Firstly, easy release of cytosolic troponin pool in non-coronary-related situations might complicate differential diagnosis of MI. Moreover, in patients with renal dysfunction troponin I as well troponin T elevations are found that cannot be linked to myocardial injury [2]. Finally, clinical practice would profit from increased sensitivity of these assays, in particular directly after symptom onset. Thus, search for new MI biomarkers remains clinically relevant task. (See Fig. 1.)

We have chosen titin as a biomarker candidate for the MI diagnosis. Titin being the largest protein discovered so far is tightly integrated into the striated muscle lattice, thus leakage of this protein into bloodstream would require myofibrillar degradation and not only a transient muscle stress. Indeed it was shown that the metalloproteinase cleaved titin fragment could act as a marker for skeletal muscle atrophy [3,4] or pathologic cardiovascular events [5]. However, in the aforementioned study a decameric oligopeptide common for both skeletal and cardiac titin isoforms was chosen as an analyte. Also, peptides less then 5 kDa are very efficiently eliminated trough kidneys resulting in short plasma half-life of few minutes.

Based on this proviso we have developed a prototype sandwich-type ELISA for the complete cardiac titin specific N2B region (98 kDa, residues 3500–4373 in UniRef Q8W242). The N2B exon is included in cardiac titin isoforms only, because it is skipped by exon skipping in skeletal muscle tissues [6]. Antigen-purified polyclonal antibodies produced in rabbit and goat (available also from www.myomedix.com) were used for capture and detection, respectively. This prototype assay was tested on sera from non-ST segment elevation (NSTEMI), STEMI patients and healthy controls (Table 1). Patient blood samples used in this study were collected at presentation to the cardiac intensive care unit. All the patients underwent a clinical assessment that included medical history, physical examination, 12-lead ECG, continuous ECG monitoring, pulse oximetry, standard blood test, chest radiography and an echocardiography. MI was diagnosed and classified according current guidelines [7,8]. The study was approved by Lithuanian Bioethics Committee (Protocol number 158,200–2011/09) and conducted in compliance with the Declaration of Helsinki.

A one-way ANOVA was conducted to determine if the log-transformed N2B serum levels were different in MI patient and control groups. Neither the presence nor absence of 3 outliers had an effect on statistical conclusion validity, thus one-way ANOVA presented here was performed after excluding outliers. Data was normally distributed for each group and there was homogeneity of variances. N2B serum levels were significantly different between different patient groups, $F(2, 95) = 19{,}966$; $p < .0001$, $\Omega^2 = 0.28$. N2B serum levels were highest in STEMI ($1.307 \pm 0.23$ log(ng/mL)), followed by NSTEMI ($1.16 \pm 0.40$ log(ng/mL)) and lowest in control group ($0.70 \pm 0.04$ log(ng/mL)). Tukey post hoc analysis revealed that the increase in N2B serum levels in STEMI (0.60, 95% CI (0.38 to 0.83), $p < 0.0001$) and in NSTEMI (0.46, 95% CI (0.22 to 0.70), $p < 0.0001$) when compared to control group was statistically significant. Assay demonstrated outstanding sensitivity and

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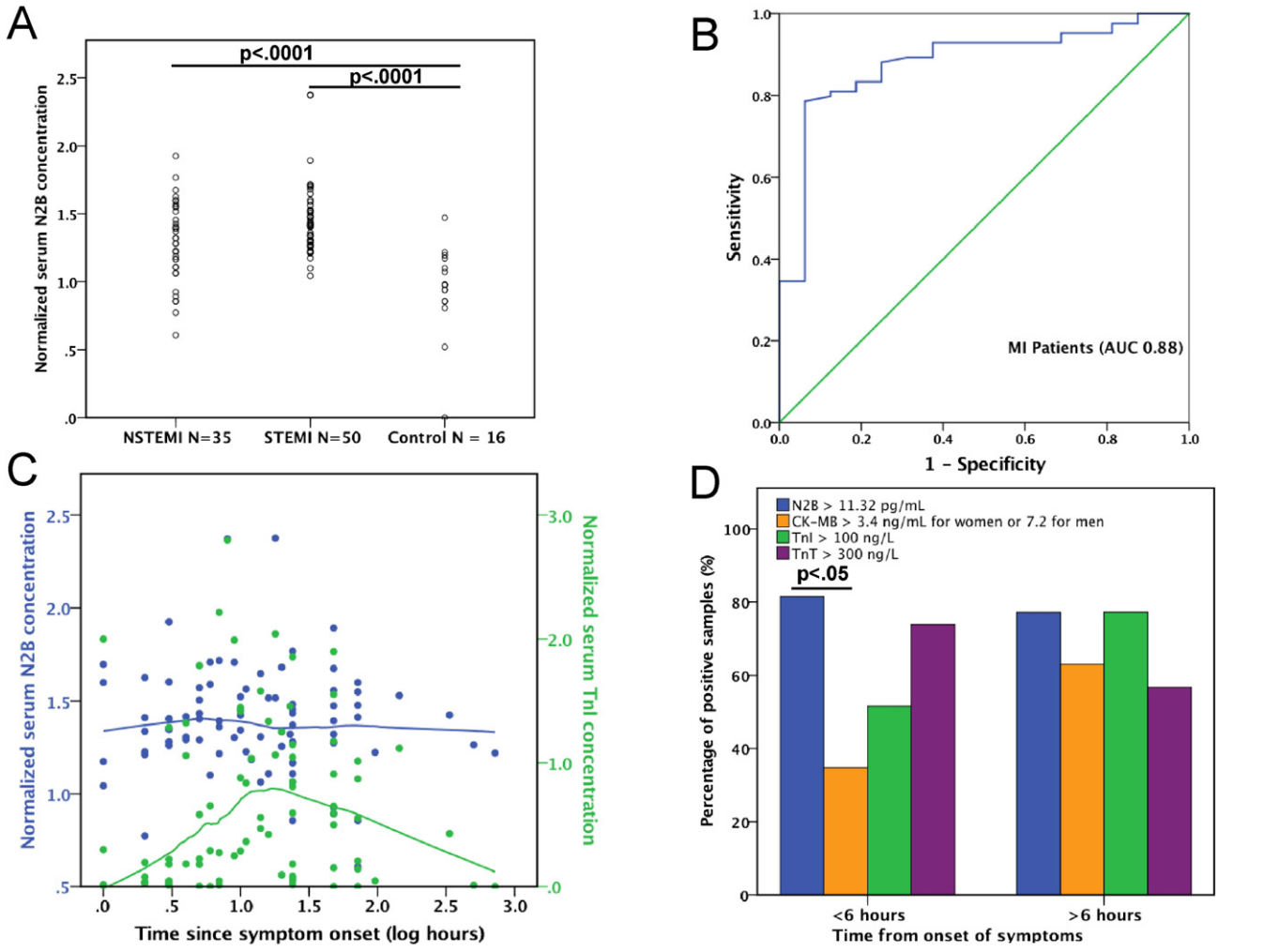


**Fig. 1.** Heart-specific N2B fragment of titin is a sensitive serological marker for cardiomyocyte injury in MI. A. Distribution of N2B serum levels in STEMI, NSTEMI patients and control sera. A one-way ANOVA revealed significant differences between groups (p-value < 0.001) and post hoc comparisons using the Tukey post hoc test revealed that STEMI and NSTEMI had significantly higher serum N2B levels than control sera. **B.** ROC curve for the developed assay. Serum N2B levels can be used as a sensitive and specific tool in diagnosing MI. **C.** Dynamics of N2B and TnI biomarkers. Measured biomarker values were plotted against the transformed time from onset of symptoms. Smoothing line for each maker was drawn to emphasise the differences in temporal dynamics of studied biomarkers. Note the time-independent nature of N2B levels in MI patients, in contrast to the single peak shaped distribution profile of TnI levels. **D.** Comparison of assay sensitivity in early and late detection of MI. Patient sera were divided into early (< 6 h) and late (> 6 h) MI samples according the time from the reported onset of symptoms and classified as positive or negative, based on the reference values for the MI diagnosis (either manufacturer reported MI rule-in criteria or 99th percentile of healthy population). N2B has comparable sensitivity to hsTnT in early MI diagnosis, and outperforms this assay in late diagnosis (explanations in text).

specificity in distinguishing MI (B), at the cut-off of 11.32 ng/mL, with 78.6% sensitivity and 93.7% specificity. This assay was as well efficient in recognising NSTEMI (AUC: 0.788; 62.9% sensitivity, 93.7% specificity) or STEMI (AUC: 0.946, 89.8% sensitivity and 93.7% specificity).

Next we evaluated how this assay performs in respect to other clinical biomarkers for heart damage. Serum N2B levels moderately correlated with serum TnI values ($r = 0.479$, $p > 0.0001$, $N = 83$) and MB-CK ($r = 0.41$, $p > 0.001$ $N = 69$), but not with hsTnT and BNP levels.

Distribution of serum N2B values in respect to time from onset of symptoms revealed rather flat profile (Panel C), whereas TnI and MB-CK (data not shown) where peaking around 10–18 h after patient reported onset of symptoms. This raises a potential use of developed assay for the early recognition of MI. To investigate this, patient cohort was divided according the time from onset of symptoms to blood sampling into early diagnosis (≤6 h) and late (>6 h) groups. Specimens were classified into positive and negative according the clinical MI rule-in criteria. Cut-off value for N2B (11.32 pg/mL) was chosen from ROC analysis. Sensitivity of developed test was compared using McNemar's test. N2B outperformed MB-CK in early diagnosis of MI (34.8% vs. 81.5%, p-value 0.012) and showed a trend towards the higher sensitivity than TnI (p-value 0.065). Noteworthy N2B test was more often positive in late MI diagnosis then hsTnT (p-value 0.096) (Panel D).

The data suggest a clinical usefulness of detection of cardiac specific titin fragment in blood as a marker for the myocardial damage. Using developed prototype assay we could demonstrate a significant elevation of biomarker in both NSTEMI and STEMI patient groups when compared to non-diseased individuals. The assay showed outstanding ROC characteristics for all studied subgroups. Obtained results indicate that serum levels of N2B might have different kinetics, than troponin T upon myocardial injury. Possibly, because titin is a very large protein and released titin N2B fragments and released titin fragments rapidly accumulate during kidney failure. Clearly, more studies are needed on the nature of titin fragmentation during myocardial necrosis.

We also noted that percutaneous coronary interventions were more often applied in NSTEMI patients with elevated N2B levels (92% *vs.* 61%, p-value 0.028 $\chi^2$-test), whereas no other tested biomarker had a statistically significant relation to PCI. If not only correlative, this might indicate that elevated titin N2B warrants PCI. Clearly, investigating larger variety of MI patients sera from different clinical settings is needed to further address the relevance of this pilot data set.

**Table 1**
Clinical characteristics of patients studied.

| | | NSTEMI | STEMI | Control |
|---|---|---|---|---|
| Sex | Male | 10 | 10 | 9 |
| | Female | 25 | 40 | 7 |
| Age years | | 64,6 ± 11,6 | 66,6 ± 13 | 45,8 ± 12 |
| N2B pg/mL | | 19,2 ± 16,7 | 31,7 ± 43,6 | 5,6 ± 6,5 |
| BMI kg/m$^2$ | | 26,8 ± 4,4 | 27,2 ± 3,9 | |
| Arterial hypertension | | 80.0% | 67.3% | |
| Diagnosed coronary diseasse | | 23.0% | 12.2% | |
| Dyslipidemia | | 89.0% | 69.4% | |
| Smoking | | 14.0% | 32.7% | |
| Previous acute coronary syndrome | None | 27 | 41 | |
| | Angina pectoris | 0 | 4 | |
| | Myocardial infarction | 6 | 4 | |
| | Unstable angina pectoris | 2 | 0 | |
| Previous acute coronary intervention | None | 31 | 44 | |
| | Coronary artery bypass surgery | 1 | 1 | |
| | Percutaneous coronary intervention | 3 | 4 | |
| Time from onset of symptoms (median hours) | | 24 | 9 | |
| TnT pg/mL | | 914 ± 1870 | 904 ± 1389 | |
| CK-MB ng/mL | | 21,8 ± 44 | 54,9 ± 101 | |
| BNP pg/mL | | 415 ± 537 | 604 ± 846 | |
| Coronarography findings | Not perfomed | 0 | 1 | |
| | No pathology | 1 | 0 | |
| | one-vessel coronary disease | 14 | 14 | |
| | two-vessel coronary disease | 6 | 14 | |
| | three-vessel coronary disease | 9 | 14 | |
| | left main trunk disease | 2 | 6 | |
| | coronary sclerosis | 3 | 0 | |
| Thrombolysis | | 0.0% | 22.4% | |
| Percutaneous coronary intervention | | 68.6% | 95.9% | |

## Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

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