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# Evaluating hs-cTnI serum levels and cTnI gene expression compared with cardiac nuclear scan in patients with angina pectoris

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**ABSTRACT**

Exercise stress can cause reversible myocardial ischemia in people with coronary artery disease (CAD). On the other hand, the new troponin biomarker with high sensitivity can detect faster and small amounts of troponin in blood circulation. The present study aimed to investigate the serum troponin level following exercise stress and the result of nuclear heart scans as the gold standard. For this purpose, 93 patients with stable angina and no history of known CAD and organic disease were included in this cross-sectional study. The serum level of the highly sensitive cardiac troponin I (hs-cTnI) was measured 75 minutes after the peak of the exercise test and reached at least 85% of the maximum heart rate. It was compared with the rate of reversible myocardial ischemia based on the nuclear heart scan, the three-month prognosis and the persistence of chest pain were investigated. Also, the expression level of the cTnI gene was evaluated by real-time PCR technique. The results showed that the average age of the patients was 58.9+12.4 years, and 62 (66.66%) patients were female. Reversible myocardial ischemia was observed in 31 patients. The relationship between hs-cTI level and the rate of reversible ischemia cases was significant (p = 0.0041). Also, the cTnI gene expression showed the same results as the serum level. According to the heart nuclear scan report, the hs-cTnI value above 1.6ng/dl had a specificity of 72% and sensitivity of 66%, a positive predictive value of 53%, and a negative predictive value of 78%. There was no significant relationship between hs-cTnI level and prognosis and the continuation of chest pain in patients after three months. Generally, the serum level of high-sensitivity cardiac troponin was higher after exercise in the group with reversible myocardial ischemia.

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## Introduction

Coronary artery disease is the leading cause of death in the world. Accurate diagnosis and timely examination of coronary artery disease (CAD) in patients with chest pain can reduce the mortality caused by it (1, 2). The exercise electrocardiography test is the primary test for diagnosing and investigating CAD, but its limitation is sensitivity and moderate specificity. Also, a nuclear heart scan is known as one of the suitable tools for diagnosing CAD (3). Still, this test, like coronary angiography, has limitations such as high cost, limited access, and radiation exposure. Among the biomarkers, cardiac troponin has been proposed as the gold standard for diagnosing myocardial infarction. The new high-sensitivity cardiac troponin I (hs-cTnI) test can detect small amounts of troponin in the blood circulation, which we could not see in the past (4). During heart attack (irreversible myocardial ischemia), the hs-cTn level in plasma increases, however, the hs-cTn level in plasma may also increase due to reversible myocardial ischemia caused by exercise stress. Previously, studies have been conducted on the relationship between high-sensitivity troponin levels and the rate of reversible myocardial ischemia, which had conflicting results (5). In the study by Sou *et al*. (6) and Sabatine *et al*. (7) the hs-cTn serum level was higher in the group with reversible myocardial ischemia. However, in the study by Lanza *et al*. (8) and the recently published meta-analysis, the level of hs-cTn was not higher in the group with reversible myocardial ischemia. In many studies, myocardial ischemia has been used as the gold standard in Single photon emission computed tomography-myocardial SPECT-MPI (perfusion imaging) (9, 10). In the present study, this method was used as a golden standard. The reasons for choosing this method go back to the greater acceptability of nuclear heart scanning due to its non-invasive process, the sufficient number of patients referred to the nuclear scanning center, and the possibility of investigating the physiological reversible myocardial

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ischemia (compared to coronary angiogram or CAG).

In the present study, hs-cTnI serum level was measured using an exercise test with treadmill ergonomics as stress to measure the reliability of high-sensitivity troponin compared with the cardiac nuclear scan as the gold standard in patients with chest pain who had a history of new stable angina without any history of CAD. Since the cTnI gene provides instructions for making a protein called cardiac troponin I, which is found solely in the cardiac muscle (11), the expression of this gene was also considered for more evaluation.

## Materials and Methods

### Patients

In this cross-sectional study for diagnosis, patients with chest pain were first visited by a cardiologist after obtaining informed consent from the patients. While reviewing the medical documents, the researcher made a preliminary checklist for the patients, including the variables related to the risk factors of the exercise test, such as hypertension, hyperlipidemia, suffering from diseases, diabetes, smoking, positive family history of heart disease and other prohibitions of performing the exercise test for the patients. Also, the number of ejection fractions of the patients was related to the echocardiography of the last month, which was performed by the patient's doctor and recorded in the patient's questionnaire.

Inclusion criteria included typical stable chest pain with all three measures of Ritter and sternal pain increasing with activity and decreasing with rest or nitrates. The non-entry criteria include not having Left bundle branch block (LBBB) or the presence of pathological Q wave CAD, previously known essential diseases such as cancer, liver disease, chronic kidney disease, end-stage renal disease (ESRD), hypertrophic cardiomyopathy, Dilated cardiomyopathy (DCM) and heart failure. Also, the non-use of beta-blocker, Blogger channel calcium, and long-acting nitrates in the last 12-48 hours, and the absence of absolute and relative contraindications for exercise testing.

Exclusion criteria include the impossibility of completing the exercise test during this test, the absolute and relative indications of stopping the exercise test, and the occurrence of any factor that prevented the continuation of the exercise test or prevented the patient from reaching at least 85% of the maximum heart rate.

### Cardiac nuclear scan

The sports stress test of qualified patients was performed on a treadmill in a multi-stage manner according to the modified Bruce protocol (12). During each stage, the speed and incline of the treadmill increased gradually. The patients ran for 6-8 minutes, and the goal was to reach the stress peak and at least 85% of the maximum heart rate, systolic and diastolic blood pressure, and how the heart waves were measured and recorded by performing an Electrocardiogram (ECG) before the test and every minute. According to the 2016 American Society of Nuclear Cardiology (ASNC) nuclear imaging clinical guide (12), patients ran on a treadmill for at least 6 minutes to prepare for a nuclear heart scan, even if they reached 85% of the maximum heart rate (Max HR).

Following achieving the maximum heart rate target and at least 6 minutes from starting the radioisotope test, 99mTc-MIBI was injected intravenously with Bq 350-250 based on weight. Patients continued to run on the treadmill for another 2 minutes after receiving the radioisotope to maintain at least 85% of Max HR. Forty-five minutes after the stress stage and receiving radiopharmaceuticals, the patients underwent gamma scanning imaging. The next day, 45 minutes after receiving the radiopharmaceutical isotope 99mT_MIBI, imaging was performed in the Representational state transfer (REST) stage. Seventy-five minutes after the end of the exercise test phase, the blood sample was taken from the antecubital vein of the patients and transferred to the laboratory to be kept at 80°C. All tests were performed by a person blind to the study in the laboratory with a viDas@hsTnI troponin kit from BioMérieux with LOT = 1006879270.

Raw SPECT-MPI images in three directions Short-axis, Vertical-long-axis, and Horizontal long-axis reconstructed the anatomical shape of the heart. Based on the semi-quantitative model, the myocardium was divided into 17 segments, and each segment received a score of 0-4 according to the amount of myocardial perfusion. After that, the summed rest score (SRS), Summed stress score (SSS) and summed difference score (SDS) were calculated. Patients with SRS < 4 were considered permanent perfusion defects. The report of the nuclear heart scan of patients based on the risk score of cardiac events as normal (SSS < 4), low risk (SSS = 4-7), medium risk (SSS = 8-12) and high risk (SSS > 12) class. Also, any segment with SDS>2 was considered reversible and exposed to myocardial ischemia. Left ventricle ejection fraction (LVEF) was calculated in the REST phase by quantitative SPECT software. Finally, the results of SPECT-MPI of the patients were reported blinded by a nuclear medicine specialist.

The results of the nuclear scan of all patients were reviewed, and patients with moderate and higher risk were advised to refer to a cardiologist. Patients were followed up after three months through phone calls or face-to-face visits.

### The hs-cTnI serum levels measurement

Blood hs-cTnl was measured in 100ng/dL. A serum level of hs-cTnI above 100ng/dl was considered as a heart attack, and none of the samples were in the range of a heart attack.

### The cTnI gene expression

The blood sample was taken from patients for RNA extraction. Blood samples were centrifuged at 400g and a temperature of 4°C for 5 minutes, and the aqueous phase was separated. 600µl of lysis buffer was added to the solution to which mercaptoethanol. Then 450 microliters of 96% ethanol were added. Seven hundred microliters of the prepared solution were transferred to the purification column and centrifuged at 12000 g for one minute. Then, 700 microliters of wash buffer one was added to the amount specified in the kit and centrifuged at 12,000 g for 1 minute, and the solution collected in the tube was discarded. Then, 600 microliters of wash buffer 1 were added to the amount specified in the kit and centrifuged at 12,000 g for 1 minute, and the solution collected in the tube was discarded. Again, then 250 microliters of wash buffer 1 were added to the amount determined in the kit and centrifuged at 12,000 g for 1 minute, and the solution collected in the tube was discarded. The extracted RNA

**Table1.** Primer sequences, product length, and annealing temperature for the cTnI gene and reference gene.

| Gene | Primer Sequence (5'-3') | Product length | Annealing temp. |
|---|---|---|---|
| GAPDH | F: AAGGGCCCTGACAAGAACTCTTT | 120bp | 55°C |
| | R: CTCCCCTCRRCAAGGGGTCG | | |
| cTnI | F: CTCTGATGCTGCAGATTGCG | 146bp | 58°C |
| | R: CTGCCGGCARAGGTCCTGAA | | |

sample was mixed with buffer and DNAase enzyme. After mixing with the solutions, it was incubated for 30 minutes at 37°C with gentle shaking to destroy the possible DNA in the sample. The obtained RNA was stored at -20 to -70 degrees Celsius.

In the next step, 1% agarose electrophoresis gel was used to measure the purified DNA. First, 0.26 g of agarose was mixed with 30 ml of TBE1X buffer and heated until completely dissolved. After some time, the gel was sufficiently cooled (temperature 40-50 degrees Celsius) and was slowly poured into the electrophoresis container containing the comb. After 30 to 40 minutes, the hardened gel was ready for DNA loading. Then five microliters of purified DNA were mixed with one microliter of loading buffer and loaded into the gel wells. Then, the current intensity of 80 milliamps and the potential difference of 90 volts was set in the electric current converter device, and the test was performed in 60 minutes. After the experiment, the band was observed using the band detector, and the gel was photographed using the GelDoc device. Finally, the bands created from different samples were analyzed in the gel. RNA samples are expected to see two bands, S18 and S28. But as said in the cDNA synthesis, a sharp band is expected in the DNA assay, which is a PCR product.

The RT reaction was performed in a final volume of 20 microliters in microtubes free of RNase and DNase. 1μg of total RNA was mixed with 30 pmol reverse primer and 10 μg of RNase-free distilled water. The above mixture was heated for 5 minutes at 70 degrees Celsius and immediately placed on ice. Then other RT reaction materials, including four microliters of RT buffer (5X), 200M- 40U enzyme, 40U RNase inhibitor, micromol dNTPS MULV, and water, were incubated for one hour at 42°C. To inactivate the enzyme for 5 minutes, 95°C was applied. The RT product was kept at -20°C until PCR.

The cTnI gene sequence was extracted from the NCBI website. Then the primer design was done with Oligo7 and Primer3 software. After manually modifying the primer using the mentioned software, the result was blasted on the NCBI website to confirm the specificity and binding of the primer to all three types. The obtained primers for the cTnI and GAPDH genes (as reference genes) are listed in Table 1.

**Statistical analysis**

The resulting data were entered into SPSS software (version 21, IBM Corporation, Armonk, NY) and analyzed as descriptive and analytical data. Using logarithmic values of hs-cTI concentration, a logarithmic transformation of values was performed. All statistical analyses were performed using logarithmic values of hs-cTnI concentration, although raw data are shown in the text. Comparison between groups of basic continuous variables was made using the Independent t-test, while the proportions were compared with the Chi-2 test. Also, the comparison of hs-cTnl level with reversible myocardial ischemia was investigated again with univariate and multivariate analyses.

## Results

Of the 472 patients with chest pain after visiting a cardiologist, 89 met this study's inclusion criteria. Four patients suffered STEMI (ST Elevation Myocardial Infarction) during the exercise stress test, and seven patients who did not reach 85% Max HR were also excluded. Finally, 93 patients were included in the study. The average age of the patients was 58.9 ± 12.4 years, and 71 (77.17%) were female. 62 (66.6%) of the patients had normal nuclear scan results. Thirty-one patients (33.4%) were classified in the group with reversible myocardial ischemia, of which 23 patients had mild involvement, three patients had moderate involvement, and 3 had severe involvement. Age, history of diabetes, blood lipids, blood pressure, smoking, and family history had no significant relationship with nuclear scan results. Myocardial ischemia was observed in 59% of the male population compared to 26% of the female population. In other words, male patients had a higher probability of reversible myocardial ischemia than females. The indexes of the fields of patients with normal and abnormal heart nuclear scan results are shown in Table 2.

The average hs-cTnI level was generally 3.04 ± 3.08 ng/dl. The average level of this serum in men was higher than in women, and the cTnI gene expression had the same results as the serum level. None of the sex, age, and risk factors, such as diabetes, hypertension, hyperlipidemia, family history, and smoking, had a significant relationship with hs-cTnI level. The relationship between age, gender, and troponin was rechecked with univariate analysis, which was again insignificant (Table 3).

The report of the nuclear heart scan of the patients was classified according to the risk score of cardiac events. The average level of hs-cTnI in cases of the standard report scan was about 2.101 ± 1.26ng/dL. In cases of mild risk in the report, the scan was about 5.1±4.3ng/dl. In cases of moderate risk in the scan, the report was about 11.7 ± 7.5ng/dl, and in extreme risk in the scan, the report was about 3.3±1.9ng/dl. In the present study, hs-cTnI blood level after peak exercise had a significant relationship with SRS (heart attack rate was not statistically significant, but with SSS and SDS) and the rate of reversible myocardial ischemia (Table 3). The obtained results were confirmed by multivariate analysis. After three months of follow-up, no cases of death or heart attack were observed in patients, but 3 cases of percutaneous coronary intervention (PCI) and 3 cases of hospitalization were reported, and no significant relationship between hs-cTn level and the three-month prognosis was observed for patients In these follow-ups, 46 patients (49.46%) complained of chest pain, and no significant correlation was seen between the nuclear scan result or troponin level with the continuation

**Table 2.** The result of nuclear heart scans with background characteristics of patients.

| Variable | Negative Nuclear Scan | | | Positive Nuclear Scan | | | P-Value |
|---|---|---|---|---|---|---|---|
| | Men | Women | P-Value | Men | Women | P-Value | |
| | Mean ± SD | Mean ± SD | | Mean ± SD | Mean ± SD | | |
| Age | 54.1± 10.9 | 58.8± 8.8 | 0.196 | 64.5± 12.1 | 61.6±12.8 | 0.687 | 0.059 |
| Hs-cTnI Level | 2.4±2.3 | 2.0±1.2 | 0.512 | 4.7±1.9 | 5.3±5.3 | 0.618 | <0.001 |
| cTnI Gene Exp. | 1.8±0.9 | 1.1±0.6 | 0.571 | 2.1±1.1 | 3.9±1.7 | 0.707 | <0.001 |
| LVEF | 50.9±7.1 | 58.0±5.2 | 0.008 | 54.2±5.5 | 57.8±2.8 | 0.152 | 0.672 |
| LVEF (Nuclear Scan) | 57.8±4.2 | 60.2±1.3 | 0.005 | 56.1±4.4 | 60.1±0.8 | 0.001 | 0.001 |
| | Number | Number | | Number | Number | | |
| Hs-cTnI<1.6 ng/ml | 8 | 31 | 0.602 | 5 | 6 | 0.933 | 0.001 |
| Hypertension | 6 | 29 | 0.733 | 7 | 10 | 0.601 | 0.481 |
| Diabetes | 1 | 15 | 0.346 | 4 | 9 | 0.141 | 0.324 |
| Hyperlipidemia | 4 | 25 | 0.421 | 4 | 10 | 0.122 | 0.430 |
| Smoking | 3 | 1 | 0.001 | 5 | 0 | 0.001 | 0.231 |
| Family Disease History | 3 | 9 | 0.671 | 13 | 2 | 0.512 | 0.359 |

Note: Hs-cTnI: High sensitive cardiac troponin I; LVEF: Left ventricle ejection fraction based on echocardiography

**Table 3.** Comparison of high sensitive cardiac troponin I (Hs-cTnI) level with the result of nuclear heart scan.

| Variable | Hs-cTnI < 1.6 ng/ml | | | Hs-cTnI > 1.6 ng/ml | | | P-Value |
|---|---|---|---|---|---|---|---|
| | Nuclear Scan (-) | Nuclear Scan (+) | P-Value | Nuclear Scan (-) | Nuclear Scan (+) | P-Value | |
| | Mean ± SD | Mean ± SD | | Mean ± SD | Mean ± SD | | |
| Age (year) | 58.8±8.9 | 59.3±11.8 | 0.793 | 53.2±13.6 | 64.2±11.3 | 0.021 | 0.762 |
| LVEF (Nuclear Scan) | 60.4±2.3 | 59.4±2.7 | 0.191 | 60.9±2.8 | 58.1±4.7 | 0.009 | 0.052 |
| Total rest score | 1.2±1.1 | 2.2±1.5 | 0.001 | 1.3±1.2 | 2.6±2.1 | 0.011 | 0.063 |
| Total stress score | 1.3±1.2 | 7.6±4.8 | 0.001 | 0.0±0.0 | 8.0±4.9 | 0.001 | 0.003 |
| Summed differential score | 1.1±1.1 | 6.5±3.7 | 0.001 | 1.2±1.4 | 6.7±3.8 | 0.001 | 0.002 |
| | Number | Number | | Number | Number | | |
| Male | 8 | 5 | 0.206 | 3 | 10 | 0.042 | 0.611 |
| Female | 32 | 6 | 0.206 | 15 | 12 | 0.042 | 0.611 |
| Hypertension | 22 | 4 | 0.303 | 13 | 14 | 0.566 | 0.291 |
| Diabetes | 10 | 5 | 0.488 | 6 | 9 | 0.606 | 0.401 |
| Hyperlipidemia | 18 | 4 | 0.811 | 9 | 7 | 0.241 | 0.707 |
| Smoking | 3 | 2 | 0.622 | 1 | 3 | 0.222 | 0.743 |
| Family Disease History | 8 | 3 | 0.901 | 4 | 2 | 0.234 | 0.267 |

Note: Hs-cTnI: High sensitive cardiac troponin I; LVEF: Left ventricle ejection fraction based on echocardiography

of chest pain.

The relationship between the level of hs-cTnI and the result of a positive nuclear scan was measured with the ROC (Receiver operating characteristic) diagram. Finally, in cases of a positive scan test, the area under the curve, or AUC, a nucleus with high hs-cTn values was 1.6ng/dl has 70% specificity and 69% sensitivity. Also, the positive predictive value of hs-cTnI > 1.6ng/dl for a positive nuclear scan result was 55%, and the negative predictive value < 1.6 ng/dl for a negative nuclear scan result was calculated as 80%. The results indicated a high negative predictive value of the hs-cTI test. If we only consider the results of a significant positive nuclear scan in cases of moderate and severe involvement that require further investigation with invasive methods such as coronary angiography, the negative predictive value of hs-cTnI <1.6ng/dl for a positive nuclear scan result will be Significant at 97% and sensitivity at 80%. Therefore, a negative hs-cTnI test can prevent additional measures.

## Discussion

In the present study, after visiting all patients with a cardiologist and taking into account the detailed clinical criteria described in the study methods, only patients with a history of stable angina and a high pretest probability for CAD were included. But in most similar studies, the previous sample population had a low probability percentage, and only the studies by Lanza *et al*. (8), which were conducted on patients with unstable angina, and the study by Axelsson *et al*. (13), which included patients with known CAD, had a high percentage of probability. The-

refore, the results of these studies have more value than similar studies. To prepare for examining myocardial ischemia with a nuclear heart scan, two different methods of exercise stress and drug stress can be applied, and exercise stress is preferable to drug stress. On the other hand, doing exercise stress with treadmill ergonomics is more physiological than bicycle ergonomics, and the present study used this method. Still, most previous similar studies used drugs or exercised stress with two-cycle ergonomics. Two studies by Lanza *et al*. (8) and Axelsson *et al*. (13) have acted like the present study. The recent survey's findings showed that the level of hs-cTnI after the peak of exercise stress in the group with reversible ischemia was higher than in the group without reversible ischemia. Still, the level of hs-cTnI after the rise of exercise stress was not related to the severity of reversible myocardial ischemia. The hs-cTnI level was not increased in fixed myocardial perfusion defect in MPI (Myocardial perfusion imaging).

In the comparison of troponin levels with reversible myocardial ischemia, the studies of Sou et al. (6), Sabatine et al. (7), Kurz et al. (14), and Wongpraparut et al. (15) showed an increase in hs-cTn level with reversible myocardial ischemia. In the study by Holder et al., the level of high-sensitivity troponin did not increase following stress and reversible myocardial ischemia (16). The reason for the different results in different studies can be seen in the studied sample population in terms of the probability of CAD, as well as the other kind of drug or sports stress used, and even the different time intervals of troponin measurement between 04 hours in the nuclear heart scan. In the conducted studies, different gold standards for hs-cTn, and a comparison of hs-cTn levels in patients with chest pain have been considered. For example, in the studies of Lanza et al. (8), Liebetra et al. (17), and Axelsson et al. (13), coronary angiography was used as the gold standard. Hs-cTn level was checked after the exercise stress test, and coronary angiography was performed on all patients. The results showed that combining troponin checks with exercise tests improves ET's sensitivity, specificity, and positive and negative predictive value.

In the study by Axelsson et al., hs-cTnT level after exercise test was compared in patients with known CAD compared to patients without CAD, the level of hs-c was higher in the group with known CAD (13), but Unlike the previous two studies, in the survey by Lanza et al., the level of hs-cTnI after stress in obstructive and non-obstructive CAD based on CAG was similar (8).

In a recent study, the negative predictive value of hs-cTI > 1.6 ng/dl for a positive nuclear scan result was 80%, and the sensitivity was 69%. In Holder et al.'s study, the negative predictive value of hs-cTnI > 1.55 ng/dl for a positive nuclear scan result was 85%, and the sensitivity was 95%, which in both studies shows the high value of low troponin level with increased sensitivity to rule out reversible myocardial ischemia (16). In a meta-analysis review article in 2019, a relationship between the level of stress and the rate of reversible myocardial ischemia was not shown. Still, the level of hs-cTn was higher following myocardial ischemia induced by exercise stress than pharmacological stress (2). In the present study, approximately half of the patients complained of chest pain after three months. No significant correlation was found between the nuclear scan result and the troponin level with the continuation of chest pain. Lanza et al.'s study also examined the continuation of chest pain after six months with hs-cTnI levels during stress; there was no significant relationship between troponin level and continued chest pain (8). The most important limitation of the study was the small number of the sample population, which caused the cases of moderate to severe involvement in nuclear heart scans to be less than expected, and the relationship between troponin level and severity of involvement in mild, moderate and severe nuclear scans was not clearly observed.

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