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# Value of High-Sensitivity C-Reactive Protein, Chronotropic Index, and Heart Rate Recovery in Predicting Cardiac Iron Overload in Patients with Beta-Thalassemia Major

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ABSTRACT

**Background:** Iron-induced cardiomyopathy is the main cause of heart failure in patients with beta-thalassemia major (β-TM). Early diagnosis and timely cardiac iron overload (IO) therapy can improve patients' prognosis.

**Objectives:** This study evaluated the value of exercise test parameters and high-sensitivity C-reactive protein (hs-CRP) in detecting cardiac IO in patients with β-TM.

**Methods:** Forty β-TM patients (age range:18 – 48) were enrolled in this cross-sectional study. Serum hs-CRP was measured using ELISA. Echocardiography and exercise treadmill tests were performed. Cardiac IO was determined using cardiac T2* (CT2*) magnetic resonance imaging, and patients were divided into abnormal (CT2* < 20 ms; n = 22) and normal (CT2* > 20 ms; n = 18) groups. Statistical analyses were conducted using SPSS software. The Mann-Whitney U-test was used to assess differences between the groups. The correlations of variables were evaluated using Pearson's or Spearman's correlation analysis. Receiver operator characteristic (ROC) curves were drawn to calculate the optimum cutoff for each test.

**Results:** We found a significantly higher level of hs-CRP (P = 0.011) and lower levels of the chronotropic index (CI) (P = 0.009) and heart rate recovery (HRR) at minutes 2 – 5 (P < 0.01) in the patients with abnormal CT2*. CT2* was inversely correlated with hs-CRP (r = -0.381, P = 0.022) and positively correlated with the CI (r = 0.346, P = 0.031) and HRR at minute 4 (HRR4) (r = 0.456, P = 0.005). ROC curve data showed diagnostic values of CI (AUC = 0.80, P = 0.005), HRR4 (AUC = 0.786, P = 0.008), and hs-CRP (0.711, P = 0.033) in predicting the severity of IO. These tests showed high sensitivity (CI = 84.6%, HRR4 = 84.6%, and hs-CRP = 85.7%) but low specificity (CI = 70.6%, HRR4 = 41.2%, and hs-CRP = 53.3%) in detecting the severity of cardiac IO.

**Conclusion:** We found that hs-CRP, CI, and HRRs were significantly associated with the severity of cardiac IO. Despite high sensitivity, these markers showed poor specificity in predicting cardiac iron deposition in β-TM patients.

## 1. Background

Frequent blood transfusions remain a long-term and necessary treatment for patients with beta-thalassemia major (β-TM), a hereditary fetal anemia defined by a marked reduction or absent synthesis of the β-globin chain of hemoglobin A (1). However, repeated blood transfusions and ineffective erythropoiesis lead to severe and toxic iron accumulation, increasing the risk of complications like cardiomyopathy, liver dysfunction, and diabetes mellitus in β-TM patients (2). Cardiac iron overload (IO) increases the risk of diastolic and systolic dysfunction (3) and atrial fibrillation (4). Nevertheless, early diagnosis and iron chelating therapy can reduce cardiac IO and improve

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patients' survival (5). Therefore, the assessment of cardiac IO plays a pivotal role in managing β-TM patients. Cardiac T2* (CT2*) magnetic resonance imaging is a sensitive, reliable, and non-invasive method to evaluate cardiac IO (6). However, high cost, low availability, a challenge to perform on children, and the need for an expert radiologist for proper interpretation have restricted the use of this technique. Therefore, novel and more available modalities may be helpful in this context. Serum markers of IO (e.g., ferritin) (7, 8) and exercise tests (9) are among the methods evaluated in previous studies, but studies have provided contradictory results.

In the immune system, IO stimulates macrophages to synthesize interleukin (IL)-6 and IL-8 (10). IL-6 stimulates the production of C-reactive protein (CRP) by the liver (11). Increased circulating IL-6 and high sensitivity-CRP (hs-CRP), correlated with carotid intima-media thickness, have been shown in patients with β-TM (12). However, some studies failed to find any significant difference in circulating hs-CRP in patients with β-TM compared to healthy controls (13).

Autonomic dysfunction has been reported in some studies conducted on β-TM patients, affecting exercise properties such as peak heart rate, VO2 max, and heart rate recovery (HRR), which is the decrease in heart rate in a specified time after exercise termination (14, 15). HRR reflects increased parasympathetic activity, decreased sympathetic activity, and decreased circulating catecholamines (16). The independent association of HRR with increased cardiovascular mortality has been established (17), and HRR has been used to predict cardiovascular risk in numerous studies (18).

## 2. Objectives

The present study evaluated the possible diagnostic value of exercise parameters and serum level of hs-CRP in predicting cardiac IO in patients with β-TM.

3. Patints and Methods

### *3.1. Patient Population*

Forty patients with β-TM were enrolled in this cross-sectional study. The patients were referred to the Aboureihan Thalassemia Center of Shahid Mohammadi Hospital (Bandar-Abbas, Iran) between March and October 2018. They were receiving packed red blood cells every two to four weeks. Hemoglobin < 8 mg/dL, heart failure (class III or IV NYHA), severe valvular heart diseases, previous coronary artery disease, use of any cardiac medications, history or presentations of symptomatic arrhythmia or syncope during exercise, inability to perform treadmill exercise test, and any active inflammatory disease were considered as exclusion criteria. The Ethics Committee of Bandar-Abbas University of Medical Sciences (IR.HUMS.REC.1397.033) approved the study protocol. The protocol was explained to the patients, and written informed consent was obtained.

### *3.2. Echocardiographic Procedure*

Echocardiography was performed for the patients in the left lateral decubitus position using a Philips Affiniti 50 device (Germany) with a 2.5 MHz probe; the results were interpreted based on the 2015 guidelines (19). The interventricular septum, posterior, left atrium, and left ventricular diameters were calculated using M-MODE and in the parasternal long axis (PSLX) view. Diastolic function was evaluated using Doppler pulsed wave (PW) and tissue Doppler imaging (TDI) at the site of the medial mitral annulus by measuring early diastolic velocity (E), late diastolic velocity (A), septal wall velocity (e'), deceleration (DT) waves, and proportions of E/é and E/A. Systolic and mean pulmonary arterial pressure (PAP) was estimated considering tricuspid regurgitation or the pulmonary insufficiency jet gradient and the right atrial pressure, which was measured by assessing inferior vena cava size and the inspiratory collapse. Tricuspid annular planar systolic excursion (TAPSE) and TDI were used for right ventricular function assessment. The achieved volumes were divided by the body surface area (BSA) and normalized (20). Modified Simpson's method was used to calculate the left ventricular ejection fraction (LVEF).

### *3.3. Exercise Test*

The exercise treadmill test (ETT) was performed on the patients using a modified Bruce protocol to assess exercise duration, exercise capacity using metabolic equivalents (METS), starting heart rate, maximum heart rate, and heart rate at 1–5 min of recovery. The patients ' O2 saturation, electrocardiogram, and blood pressure were monitored during the symptom-limited test. Exercise testing was terminated if fatigue, dizziness, chest discomfort, dyspnea, or muscle cramps developed.

#### *3.3.1. Heart Rate Recovery (HRR) and Chronotropic Index (CI)*

After exercise recovery, heart rate (HR) data were recorded when the patient rested sitting. HRR was calculated by subtracting the HR value at 1 to 5 minutes (HRR1, HRR2, HRR3, HRR4, and HRR5) from the peak HR, as described by Kucukseymen et al. (18). The following formula: CI = (HR peak-HR baseline) / (220-age-HR baseline) was used for calculating the Chronotropic index (CI).CI < 0.8 was considered chronotropic incompetence.

### *3.4. Cardiac T2* Magnetic Resonance Imaging*

Cardiac T2* (CT2*) magnetic resonance imaging (MRI) was performed using a Siemens Avanto 1.5 Tesla MRI device (Siemens, Germany) at Saheb Al Zaman Hospital (Bandar Abbas, Iran). The patients were categorized as low (CT2* > 20 ms), mild (CT2*: 10 - 20 ms), or high risk ($CT2^{*}$ < 10 ms).

### *3.5. Biochemical Measurements*

Blood samples were obtained from all patients before exercise testing to determine the values of hemoglobin and hs-CRP. Sera were separated and stored at -80 ºC until testing; hs-CRP was measured using ELISA kits (Zell Bio kit, Germany) according to the manufacturer's instructions.

### *3.6. Statistical Analysis*

Statistical analyses were performed using SPSS (version

16.0, SPSS, Chicago, IL, USA). Data are expressed as mean ± standard deviation for continuous variables and percentages for categorical variables. Data were tested for normal distribution using the Kolmogorov-Smirnov test. The independent samples t-test and Mann-Whitney U-test were used to assess the differences between the groups. The correlations of variables were evaluated using Pearson's or Spearman's correlation analysis. Receiver operator characteristic (ROC) curve analysis and calculation of the area under the curve (AUC) were performed for hs-CRP, CI, and HRR2–5 in 40 patients. The optimum cutoff for each test was selected from the ROC analysis and used to classify the patients. Each test's clinical sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. A P-value < 0.05 was considered statistically significant.

## 4. Results

Table 1 shows the characteristics of forty β-TM patients (age 27.65 ± 5.99) enrolled in this study. The average Hb level was 10.92 ± 1.42 g/dL. The mean CT2* was 19.96 ± 10.48 ms. Normal (CT2* > 20 ms), mild-moderate (CT2* 10 to 20 ms), and severe myocardial iron deposition (CT2* < 10 ms) were observed in 42.5, 37.5, and 20% of patients, respectively. The level of hs-CRP was 5.68 ± 4.43 (range: 0.0 to 14.39) mg/L. According to their hs-CRP levels, the patients were grouped into three groups: 69.4% had high-risk levels (hs-CRP > 3.0 ng/mL), 13.9% had low-risk levels (hs-CRP = 1 – 3 mg/mL), and 16.7% had normal levels (hs-CRP < 1.0 mg/L). Except for one patient with LVEF below 50 percent, others had normal LV systolic function. Thirty percent of the patients revealed Diastolic dysfunction (E/é < 10).

**Table 1.** Characteristics of the Study Population of Patients with Beta-thalassemia Major

| Variable | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|
| BMI (kg/m2) | 20.55 | 3.33 | 14.04 | 30.36 |
| Age (years) | 27.65 | 5.99 | 18 | 48 |
| Hb (mg/dL) | 10.92 | 1.42 | 8.1 | 14.8 |
| CT2* (ms) | 19.96 | 10.48 | 5.11 | 44.55 |
| hs-CRP (mg/L) | 5.68 | 4.43 | 0.00 | 14.39 |
| METS | 10.31 | 2.63 | 4.6 | 16.9 |
| VO2MAX | 36.08 | 9.19 | 16.10 | 59.10 |
| Distance | 760.85 | 198.86 | 386 | 1343 |
| TE time | 12.75 | 2.32 | 7.08 | 18.34 |
| Peak HR | 168.10 | 19.71 | 114 | 206 |
| HRR1 | 29.55 | 8.75 | 12 | 49 |
| HRR2 | 63.18 | 14.01 | 22 | 90 |
| HRR3 | 70.21 | 14.38 | 27 | 97 |
| HRR4 | 73.16 | 15.31 | 37 | 101 |
| HRR5 | 76.47 | 15.71 | 37 | 111 |
| Max HR | 89.74 | 9.86 | 59 | 109 |
| CI | 0.76 | 0.17 | 0.14 | 1.09 |
| Pred METS | 12.00 | 1.61 | 8.46 | 15.30 |

Abbreviations: CT2*, cardiac T2*; hs-CRP, high-sensitivity C-reactive protein; Hb, hemoglobin; BMI, body mass index; LVEF, left ventricular ejection fraction; Max HR, maximum heart rate; METs, metabolic equivalents; HRR, heart rate recovery; CI, chronotropic index.

**Table 2.** Demographic and Clinical Characteristics of the Patients with Low and High Cardiac iron Deposition

| Variable | CT2* < 20 ms (n = 22) | CT2* > 20 ms (n = 18) | P-values |
|---|---|---|---|
| Age (years) | 28.9 ± 6.5 | 26.1 ± 5.5 | 0.144 |
| BMI (kg/m2) | 20.9 ± 3.4 | 20.1 ± 3.3 | 0.457 |
| Hb (mg/dL) | 11.1 ± 1.6 | 10.7 ± 1.2 | 0.325 |
| CT2* (ms) | 12.3 ± 4.3 | 29.3 ± 7.8 | < 0.001 |
| hs-CRP (mg/L) | 7.3 ± 4.2 | 3.6 ± 3.9 | 0.011 |
| METS | 10.1 ± 2.7 | 10.6 ± 2.6 | 0.532 |
| VO2MAX | 35.2 ± 9.3 | 37.1 ± 9.2 | 0.533 |
| Distance | 708 ± 180 | 789 ± 222 | 0.431 |
| TE time | 12.5 ± 2.2 | 13.0 ± 2.5 | 0.501 |
| Peak HR | 160 ± 17 | 177 ± 19 | 0.006 |
| HRR1 | 27.6 ± 7.1 | 31. 8 ± 9.2 | 0.139 |
| HRR2 | 58.8 ± 13.0 | 68.1 ± 13.8 | 0.04 |
| HRR3 | 64.3 ± 13.3 | 76.7 ± 12.9 | 0.006 |
| HRR4 | 66.7 ± 14.1 | 79.9 ± 13.9 | 0.007 |
| HRR5 | 69.8 ± 15.1 | 84.1 ± 13.0 | 0.01 |
| Max HR | 86.3 ± 9.3 | 93.5 ± 9.4 | 0.026 |
| CI | 0.70 ± 0.16 | 0.84 ± 0.16 | 0.009 |
| Pred METS | 11.8 ± 1.5 | 12.3 ± 1.7 | 0.393 |

Abbreviations: CT2*, cardiac T2*; hs-CRP, high-sensitivity C-reactive protein; Hb, hemoglobin; BMI, body mass index; LVEF, left ventricular ejection fraction; Max HR, maximum heart rate; METs, metabolic equivalents; HRR, heart rate recovery; CI, chronotropic index.

*4.1. Comparison of the Variables between the Patients with Low Cardiac Iron Deposition and Medium to High Cardiac Iron Deposition*

The patients were divided into low (CT2* > 20 ms) and medium to high cardiac iron deposition (CT2* < 20 ms) groups to assess the relationship between the severity of myocardial iron deposition and demographic characteristics, exercise test variables, and biochemical measures (Table 2). No significant differences existed between the groups regarding age, BMI, and Hb concentration. A significantly higher value of hs-CRP was observed in patients with CT2* < 20 ms compared to patients with CT2* > 20 ms. Furthermore, significantly lower levels of peak HR, HHR 2 - 5, max HR, and CI were found in patients with T2* < 20 ms relative to patients with CT2* > 20 ms. When the patients with CT2* < 20 ms were separated into high cardiac iron deposition (CT2* < 10) and medium cardiac iron deposition (CT2* = 10 – 20 ms), no significant difference was observed between the groups in the various variables.

*4.2. Correlation between Cardiac T2*, Chronotropic Index, and hs-CRP and Various Variables*

The correlation of CT2*, CI, and hs-CRP with various variables is summarized in Table 3. As shown, hs-CRP, Peak HR, and HRR2-5 correlated significantly with CT2*. Also, CI correlated significantly with CT2* and various exercise test parameters. The data showed a positive correlation between hs-CRP, CT2*, and HRR2-5.

*4.3. ROC Curve Analysis*

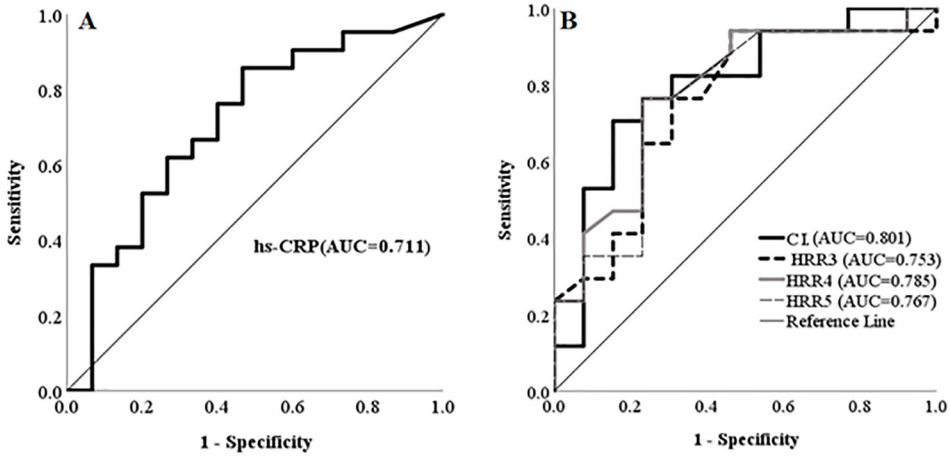
ROC curve analysis was applied to evaluate the possible performance of hs-CRP, CI, and HRR3-5 in identifying patients at higher risk of iron-induced cardiomyopathy. Figure 1 A illustrates the ROC curve of hs-CRP. The AUC for hs-CRP was 0.711 (95% CI 0.534 to 0.888). The cutoff concentration of 2.66 mg/L was determined, with a specificity of 53.3%, sensitivity of 85.7%, PPV of 72.0%, and NPV of 72.7% (Table 4). ROC curve analysis showed an AUC of 0.8 for CI (Figure 1 B), suggesting a high sensitivity of this test in predicting cardiac IO. With the optimum diagnostic cutoff point of 0.8022, a sensitivity of 84.6%, specificity of 70.6%, PPV of 72.7%, and NPV of 70.5%

**Table 3.** Bivariate Correlation between Cardiac T2*, Chronotropic Index, and High-Sensitivity CRP

| Variables | CT2* r (P) | CI r (P) | hs-CRP r (P) |
|---|---|---|---|
| CT2* | ---------- | 0.346 (0.031) | -0.381 (0.022) |
| CI | 0.346 (0.031) | ---------- | -0.261 (0.130) |
| hs-CRP | -0.381 (0.022) | 0.261 (0.130) | ---------- |
| METS | 0.100 (0.540) | 0.566 (0.001) | -0.094 (0.586) |
| VO2MAX | 0.099 (0.542) | 0.566 (0.001) | -0.094 (0.587) |
| Distance | 0.100 (0.540) | 0.616 (< 0.001) | -0.218 (0.201) |
| TE time | 0.086 (0.605) | 0.678 (< 0.001) | -0.204 (0.239) |
| Peak HR | 0.342 (0.033) | 0.966 (< 0.001) | 0.343 (0.044) |
| HRR1 | 0.293 (0.074) | 0.455 (0.004) | -0.272 (0.122) |
| HRR2 | 0.408 (0.011) | 0.651 (< 0.001) | -0.399 (0.020) |
| HRR3 | 0.477 (0.005) | 0.748 (< 0.001) | -0.478 (0.004) |
| HRR4 | 0.456 (0.005) | 0.772 (< 0.001) | -0.486 (0.004) |
| HRR5 | 0.407 (0.025) | 0.770 (< 0.001) | -0.386 (0.042) |
| Max HR | 0.299 (0.065) | 0.990 (< 0.001) | 0.147 (0.400) |

Abbreviations: CT2*; cardiac T2*, hs-CRP; high-sensitivity C-reactive protein, Hb; hemoglobin, BMI; body mass index, LVEF; left ventricular ejection fraction, Max HR; maximum heart rate, METs; metabolic equivalents, HRR; heart rate recovery, CI; chronotropic index.

**Figure 1.** Receiver Operator Characteristic Curves Comparing the Performance of hs-CRP (A), CI, and HRR3-5 (B) in Detecting the Severity of Cardiac Iron Deposition




Abbreviations: CI; chronotropic index, hs-CRP; high-sensitivity C-reactive protein, HRR; heart rate recovery.

**Table 4.** Diagnostic Characteristics of Various Markers in Detecting Cardiac Iron Deposition

| Marker | AUC | P | Cutoff | SEN (%) | SPE (%) | NPV (%) | PPV (%) |
|---|---|---|---|---|---|---|---|
| hs-CRP | 0.711 | 0.033 | 2.66 | 85.7 | 53.3 | 72.7 | 72.0 |
| CI | 0.801 | 0.005 | 0.8 | 84.6 | 70.6 | 70.5 | 72.7 |
| HRR3 | 0.753 | 0.019 | 64 | 76.9 | 52.9 | 58.8 | 80.9 |
| HRR4 | 0.785 | 0.008 | 66 | 84.6 | 41.2 | 64.7 | 80.0 |
| HRR5 | 0.767 | 0.014 | 70 | 84.6 | 47.1 | 76.9 | 76.4 |

Abbreviations: AUC; area under the curve, SEN; sensitivity, SPE; specificity, PPV; positive predictive value, NPV; negative predictive value, CI; chronotropic index, hs-CRP; high-sensitivity CRP, HRR; heart rate recovery.

were obtained (Table 4). Figure 1 B also exhibits ROC curve analysis of HRR3-5 in detecting cardiac IO. ROC curve analysis showed AUC values of 0.753, 0.782, and 0.750 for HHR3, HHR4, and HHR5, respectively. The clinical performances of these indices in detecting cardiac iron are summarized in Table 4; they showed good sensitivity but low specificity. Although combining the results of these variables with the results of hs-CRP increased the sensitivity for detecting patients at risk of IO, it reduced the specificity (data not shown).

## 5. Discussion

Early detection of iron cardiomyopathy significantly impacts the survival and prognosis of patients with β-TM. Due to the limited availability of CT2* MRI, the gold standard method for detecting cardiac IO (21), in many areas with a high prevalence of β-TM, alternative methods are necessary. The results of the present study revealed a significantly higher level of hs-CRP in the patients with higher levels of IO (CT2* < 20 ms) compared to patients with a low amount of cardiac IO (CT2* > 20 ms). Furthermore, some exercise test parameters, including HHR 2 - 5 and CI, were significantly lower in the patients with CT2* < 20 ms than with T2* > 20 ms. Correlation analysis demonstrated that CT2* correlated positively with CI and HRR and negatively with hs-CRP. ROC curve analysis showed high hs-CRP, CI, and HRR sensitivities in distinguishing patients with and without cardiac IO. However, these tests showed poor specificity compared to CT2* in predicting cardiac IO.

Elevated values of circulating hs-CRP in patients with β-TM have been documented in several studies (12, 22), suggesting a possible role of chronic inflammation in β-TM pathogenesis (23). Although the exact mechanisms are not clearly described, IO-induced oxidative stress (24) and -IL-6 production may be involved in hs-CRP upregulation (11). The mean hs-CRP level in the patients enrolled in this study (5.6 ± 4.4 mg/L) was similar to those reported previously, indicating increased values of hs-CRP in β-TM patients (25). Our data also showed a higher level of hs-CRP in the β-TM patients with abnormal CT2*, particularly those with CT2* < 10 ms, compared to those with normal CT2*. According to ROC curve analysis, hs-CRP with a cutoff value of 0.261 showed high sensitivity (85.7%) but poor specificity (50.6%) in detecting cardiac iron deposition, suggesting that circulating values of hs-CRP not only function as a marker of endothelial dysfunction (12) but also predict the severity of cardiac IO in β-TM patients.

The association of chronotropic incompetence with adverse clinical outcomes has been demonstrated in individuals with and without CVD (26). In β-TM patients, adverse effects of IO have been shown on the activity of the autonomic system and, consequently, on resting HR and CI abnormalities (27). The present study's data correlated CI positively with CT2* values, and the frequency of chronotropic incompetence was significantly higher in the patients with abnormal CT2*. Moreover, we observed a negative association between hs-CRP and CI, in line with the findings of a previous study (28).

Kucukseymen et al. explained that HRR could be a novel test to predict iron cardiomyopathy in β-TM (18). They showed lower HRR1-3 values in patients with abnormal CT2*. A similar finding was obtained in the present study, and lower HRR2-5 values were observed in the patients with abnormal CT2*(< 20 ms) compared to those with normal CT2* (< 20 ms). Our findings also revealed a marked inverse association between HRR2-5 and hs-CRP. The possible diagnostic value of CI and HRR in detecting abnormal cardiac iron deposition was evaluated using ROC curve analysis. Accordingly, these markers showed acceptable diagnostic values in detecting abnormal iron deposition. CI with AUC = .8 was superior to HRRs and, with a cutoff value of 0.8, showed high sensitivity (84.6%) but low specificity (70.6%) in detecting cardiac iron deposition. HRR3-5 showed high sensitivity but very low specificity in detecting abnormal cardiac iron deposition. Due to relatively high sensitivity, these methods can help rule out the presence of abnormal cardiac iron deposition in β-TM patients; however, none of them has diagnostic efficiency for use instead of CT2* MRI.

Although this study showed a significant association of CT2* with HRR, CI, and hs-CRP, previous studies revealed improved exercise capacity, chronotropic response, and HRR after rehabilitation programs (29). Furthermore, rehabilitation can significantly decrease hs-CRP based on the results of Milani et al. (30). The small sample size and cross-sectional nature of this study are other limitations. Therefore, further studies in a larger population of β-TM patients with long-term monitoring of HRR are needed to clarify the significance of these findings.

### *5.1. Conclusion*

This study revealed a significant difference in hs-CRP, CI, and HRR2-5 values between β-TM patients with and without abnormal cardiac iron deposition. ROC curve analysis showed remarkable sensitivity but medium to low specificity of these markers in discriminating between patients with and without cardiac iron deposition, suggesting the inferior performance of these markers in predicting cardiac IO compared to CT2*.

*5.2. Ethical Approval*

This study was approved under the ethics approval code IR.HUMS.REC.1397.033 (webpage: https://ethics.research.ac.ir/EthicsProposalView.php?id=11815)

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There is no acknowledgement.

**Authors' Contribution**

This study was conceived and designed by M. N., who also supervised echocardiograms and data collection and helped draft the manuscript. A. G. participated in designing the study, evaluated the patients, collected the clinical data, interpreted them, and drafted the initial manuscript. E. E. performed parts of data evaluation, collected laboratory data, and revised the manuscript. S. R. performed the statistical analysis and revised the manuscript. O.G. re-analyzed the clinical and statistical data and revised the manuscript. All authors read and approved the final manuscript.

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