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**RESEARCH ARTICLE**

# Assessment of Circulating CCR6 Level in Acute Myocardial Infarction and its Association with Disease Severity.

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**Abstract:** ***Background*:** Acute myocardial infarction (AMI) pathophysiology is mediated by systemic, intraplaque myocardial inflammatory processes that occur mainly due to coronary artery thrombosis in an atherosclerotic plaque area. The G-protein-coupled chemokine receptor (Ccr6) is displayed on the surface of many types of leukocytes, that have been found in atherosclerotic plaques. It is a novel mediator of inflammation and immune response.

***Objectives*:** To determine CCR6 lymphocyte expression in AMI patients and its association with disease severity using the Gensini scoring system.

***Methods*:** 25 AMI patients and 25 controls underwent flow cytometry to determine the percentage of circulating CCR6+ lymphocytes. To forecast AMI and determine how CCR6 expression relates to it, multivariate logistic regression analysis was used.

***Results and Discussion*:** There was a higher percentage of CCR6+ lymphocyte expression in AMI patients than in controls. In addition, CCR6 showed a significant positive correlation with the Gensini score (GS) in the AMI group then with the degree of coronary artery disease (CAD).

***Conclusion*:** The chemokine receptor CCR6 is an independent biomarker for AMI and mayplay a role as a mediator of T lymphocyte recruitment, which is associated with coronary lesion destabilization.

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## 1. INTRODUCTION

Cardiovascular disease is a significant contributor to morbidity, death, and disability worldwide. It is the second most prevalent cause of mortality for females whereas, for males, it is the first [1, 2]. One of the clinical indications of coronary heart disease is myocardial infarction (MI) [3] which occurs mostly due to coronary artery thrombotic occlusion in an atherosclerotic plaque that undergoes acute plaque change [4].

The pathogenesis of atherosclerosis is hallmarked by lipid accumulation, smooth muscle cell migration, cell apoptosis, necrosis, then fibrosis, and immune and inflammatory responses. Lymphocytes are crucial in the early stages of inflammation and innate immune responses. T cells are recruited to the atherosclerotic lesion *via* several adhesion molecules and chemokines [5]. Several chemokines and chemokine receptors (CCRs) are involved in the atherosclerotic process, including the inflammatory receptors CCR2, CCR5, CxCR2, and Cx3CR1, the classic homeostatic receptor CCR7, and CCR6 [6, 7].

A special subset of cytokines known as chemokines is tiny molecular weight proteins that are recognized to fulfill a range of varied functions in the human immune system [8]. T and B lymphocytes, dendritic cells, macrophages, monocytes, neutrophils, eosinophils, basophils, innate lymphoid cells, neurons, epithelial and endothelial cells all contain chemokines and their receptors extracellularly. Multiple autoimmune disorders, chronic inflammatory diseases, cancer metastasis, and human immunodeficiency virus (HIV) infection are caused by chemokine-mediated immune cell migration [9].

Leukocyte chemoattraction and cell-to-cell communication are both facilitated by the chemokine receptor 6

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(CCR6). It can activate immune cells into inflammatory tissues by its specific ligand, called CCL20 [10].

CCR6 (also known as CD196 or C-C, CKR-6) is constitutively expressed in lymphatic and non-lymphatic tissues, most prominently in the spleen, lymph nodes, appendix, and pancreas, but also to a smaller level in the thymus, colon, small intestine, fetal liver, and testis [11]. Additionally, it is present in a number of leukocyte subsets, such as immature dendritic cells (iDCs), B cells, T cells (including regulatory Treg cells and pro-inflammatory Th17 cells), NKT cells, and neutrophils [12].

In atherosclerotic lesions or autoimmune illnesses in general, CCR6 contributes significantly as a modulator of immune response and inflammation [13]. However, the CCR6 expression in patients and its relationship with AMI have not been clarified yet. According to angiographic findings, the Gensini scoring system is a relatively objective approach for determining the severity of CAD [14]. Therefore, the current study aimed to determine if circulating CCR6 levels increase in AMI patients.

## 2. SUBJECTS AND METHODS

### 2.1. Study Subjects

This case-control research had 25 patients with AMI undergoing coronary angiography **(**Group I) and 25 who presented with chestpain and other risk elements for CAD but with normal coronary angiography as a control group (Group II). Between December 2020 and February 2021, patients were enlisted from the cardiology unit at the University Hospitals of Benha.

The Benha Faculty of Medicine's local ethics committee authorized the study. Each person gave their informed consent prior to the collection of sample.

### 2.2. Sample Collection

For the AMI patients, blood samples were collected just after coronary angiography or within 48 h after performing the percutaneous coronary intervention. While for the control group, following their admission, blood samples were collected just after coronary angiography. Three milliliters of peripheral venous blood were obtained under complete aseptic conditions while the patients were fasting for 12-14 hours. The drawn sample was divided into; 1 milliliter of K-EDTA anticoagulant for CBC and assessment of CCR6 on lymphocytes by flowcytometry, and 2 milliliters for serum separation was used for measuring the lipid profile and uric acid.

### 2.3. Assessment of Coronary Artery Disease Severity Using Gensini Score

The Gensini score indicates coronary atherosclerosis severity. It was calculated after coronary angiography for each patient by identifying the relevance of location of the lesion and the diameter of the lumen reduction of every coronary artery. The lumen diameter reductions (25%, 50%, 75%, 90%, 99%, and total occlusion) were analyzed and assigned Gensini ratings of 1, 2, 4, 8, 16, and 32, respectively. This score was increased by the following factor to represent the clinical importance of the lesion's site in the coronary circulation: the left main coronary artery has a diameter of 5, the proximal segment of left anterior descending coronary artery (LAD) has a diameter of 2.5, the proximal segment of left circumflex artery (LCX) has a diameter of 2.5, the mid-segment of the LAD has a diameter of 1.5, 1 for the right coronary artery, mid distal LCX, and distal LAD.

### 2.4. Flow Cytometric Analysis of CCR6

Using flow cytometry, the percentages of CCR6+ lymphocytes were calculated. Staining was performed using fluorescein isothiocyanate (FITC) conjugated isotype control antibodies for IgG1 (BD Biosciences, USA, catalog number: 554121) and phycoerythrin (PE) conjugated isotype control antibodies for IgG2 and CCR6 (BD Biosciences, USA, catalog number: 349053). Staining was performed using two color combinations of conjugated antibodies by adding 10 µL of monoclonal antibody to 50 µL of blood in 2 tubes (the first containing anti IgG1, IgG2 with blood, and the second tube containing anti-CCR6 PE with blood). The tubes were kept at ambient temperature and incubated for 20 minutes in the dark. After incubation, FACS lysed solution was used to lyse RBCs (BD Biosciences, USA, Catalog number: 349202) for 10 minutes, then centrifugated at 1200 rpm for 5 minutes. The pellet was resuspended after the supernatant was aspirated, washed twice with 2 ml of phosphate-buffered saline PBS), then resuspended in 0.5 ml of PBS and inspected. The background fluorescence intensity was evaluated for each case using an isotype-matched negative control sample (BD Biosciences). Using established quality control techniques, the FACS Caliber flow cytometer (BD Biosciences, USA) was configured to capture stained cells. For cases and controlled types, at least 10,000 events were collected. By utilizing a FACS Calibur flow cytometer, the percentages of CCR6+ lymphocytes were calculated. The data were then processed and shown using the Cell Quest software.

### 2.5. Gating and Method of Interpretation

The gates and isotype control were illustrated in Figs. (**1**-**3**).

### 2.6. Statistical Analysis

For statistical analysis and data management, we utilized SPSS version 25 (IBM, Armonk, New York, United States). In order to determine whether quantitative data were normal, the Shapiro-Wilk test and direct data visualization techniques were used. Means and standard deviations were employed to sum up numerical data, whereas percentages and numbers were utilized to sum up categorical data. Quantitative data were compared between the research groups using an independent t-test. Using the Chi-square test, categorical data were compared. Using Pearson's correlation, correlation analyses were performed. ROC analysis was performed for CCR6 to diagnose AMI. The optimal cut-off point, diagnostic indices, and Area Under Curve (AUC) with a 95%

confidence interval (CI) were computed. MI was predicted using a multivariate logistic regression model. Calculations were made to establish the odds ratios and 95% CI. There were two sides to each statistical test. *P* values under 0.05 were viewed as significant

## 3. RESULTS

Table **1** displays the characteristics of controls and patients with AMI. No significant differences were reported between the studied groups concerning all general characteristics, including age, sex, smoking, diabetes mellitus, hypertension, and BMI.

All vital signs showed nonsignificant differences between the studied groups, including systolic blood pressure, diastolic blood pressure, heart rate, temperature, and respiratory rate. The mean Gensini score was 59 ± 24 (Table **2**).

All CBC parameters exhibited no significant changes between the study groups, including TLC, lymphocytes, monocytes, granulocytes, RBCs, hemoglobin, hematocrit, MCV, MCH, MCHC, and platelets (Table **3**).

HDLc was significantly lower in cases than in controls (P = 0.029). In contrast, total cholesterol (TC)and LDLc were higher in AMI (group I) compared to controls (group II), and triglycerides (TGs) were higher in controls (group II) compared to AMI (group I), however, because of the small sample size, statistical significance could not be attained (Table **4**).

Serum uric acid was significantly higher in cases than in controls (P = 0.018). In addition, CCR6 was significantly higher in cases (32.93%) than in controls (16.58%) ($P < 0.001$) (Table **5**).

CCR6 was positively associated with GS (r = 0.798, $P < 0.001$) (Fig. **4**). In contrast, it showed no significant correlation with age, BMI, heart rate, respiratory rate, CBC parameters, lipid profile, and serum uric acid.

ROC analysis was performed for CCR6 to diagnose MI and showed a significantly excellent AUC of 0.97 with a 95% CI ranging from 0.932 to 1.0. The best cut-off was >26.84%, at which sensitivity, specificity, PPV, and NPV were 80%, 100%, 100%, and 83.3%, respectively (Fig. **5**).

To forecast MI, multivariate logistic regression analysis was conducted. It revealed that CCR6, serum uric acid, and HDLc were significant independent predictors for MI, controlling for age, gender, DM, HTN, and smoking (Table **6**).

## 4. DISCUSSION

The current study showed significantly higher CCR6 levels in AMI patients than in controls. This finding is consistent with Shi *et al*., who reported a significantly higher percentage of CCR6+ lymphocytes in AMI patients than in controls [13]. In addition, they stated that the amount of CCR6+ lymphocytes is a distinct biomarker for AMI. According to Yan *et al*., CCR6 is a biomarker connected to the inflammatory and immunological response reaction in AMI pathogenesis because it facilitates -T-cell infiltration into an infarcted heart through the CCL20-CCR6 signaling pathway [15]. Another animal study demonstrated that the lesion in the aortic area in $CCR6^{+/+}$ $ApoE^{-/-}$ mice is greater than that in $CCR6^{-/-}ApoE^{-/-}$ mice, and the macrophage content is also higher in the lesion area of $CCR6^{+/+}$ $ApoE^{-/-}$ mice which support the role of CCR6 in atherosclerosis in ApoE-deficient mice [16].

CCR6 demonstrated a significant positive connection with the GS in this study. However, there were no significant relationships between CCR6 levels and age, BMI, heart rate, respiratory rate, or laboratory values. These results are in line with those of Shi *et al*., who found that the proportion of CCR6+ lymphocytes positively correlates with the degree of coronary artery stenosis disease as measured by the GS [13].

In the current study, all general variables, including age, gender, smoking, diabetes mellitus, hypertension, BMI, and vital signs, exhibited no statistically significant differences between the study groups. These findings are consistent with Shi *et al*., who claimed that there were no significant differences between the studied groups regarding age, sex, hypertension, diabetes, and smoking [13].

The current study showed no significant differences in CBC parameters between the groups under study. In contrast, Gunes *et al.* state that WBC, RDW, neutrophil count, and neutrophil-lymphocyte ratio (NLR) are significantly higher in patients than in controls [17]. However, they reported no significant differences in mean platelet volume (MPV), platelet distribution width (PDW), lymphocyte count, and hemoglobin. In addition, Yilmaz *et al.* claim high thrombus incidence (NSTEMI) among NST-ACS patients with elevated neutrophil and low lymphocyte counts [18]. Furthermore, Korkmaz *et al.* imply that troponin-positive patients have higher leucocytic and neutrophilic counts but lower lymphocytic counts than those patients who had a negative Troponin [19].

In this study, there was a significantly lower high-density lipoprotein (HDLc) in cases than in controls, while total cholesterol (TC), triglycerides (TG), and LDLc did not differ substantially. These results are supported by Shi *et al*., who assert lower HDLc in AMI patients than in controls but report higher LDL levels in AMI patients [13]. Additionally, Khan *et al.* state a significant decrease in HDLc levels in STEMI and NSTEMI patients compared to controls, with no difference in TG [20].

We discovered that the mean serum uric acid concentration was significantly greater in patients versus controls. This result agrees with Gosar *et al*., who reported that AMI patients have higher serum uric acid levels than controls [21]. Furthermore, Nadkar and Jain reported higher serum uric acid in patients with AMI than in healthy individuals [22]. In contrast, Shi *et al.* stated that there was no significant difference between groups in serum uric acid levels [13].

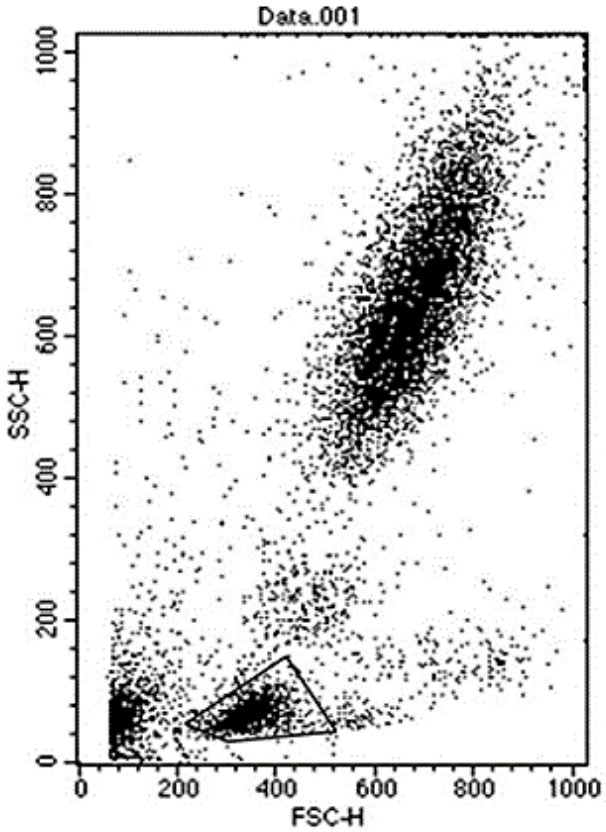


**Fig. (1).** The first gate (G1) was set on lymphocytes based on forward light scatter and side light scatter.

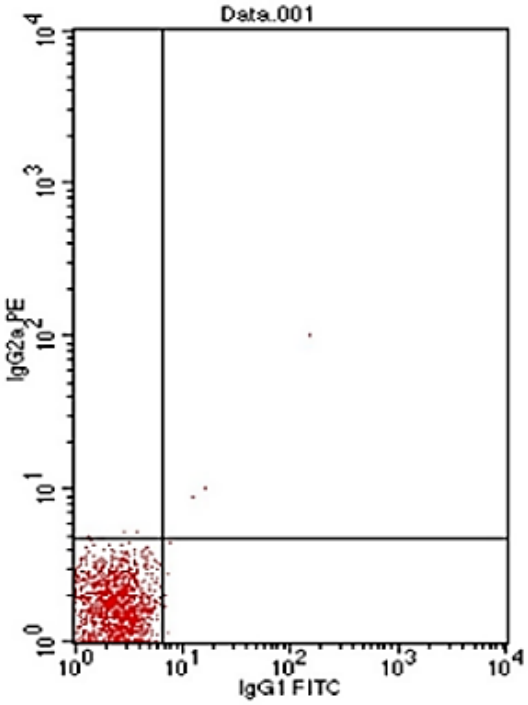


**Fig. (2).** An isotype control was used for quadrant adjustment to subtract auto-fluorescence and non-specific binding.

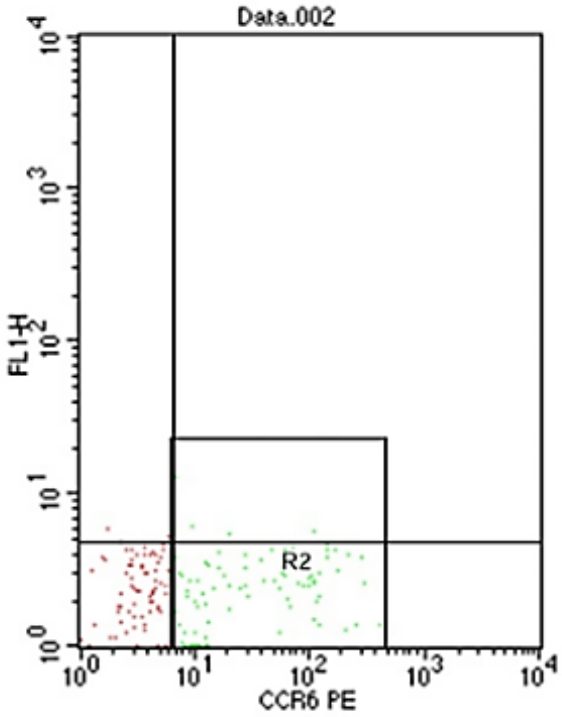


**Fig. (3).** The second gate (G2) was set on the population of lymphocytes which are positive for CCR6.

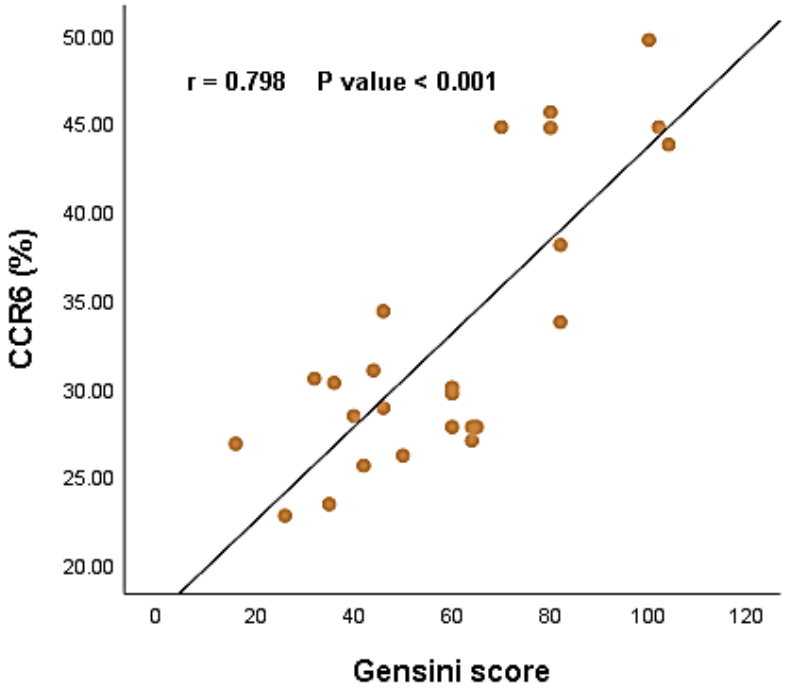


**Fig. (4).** Correlation between CCR6 and Gensini score.

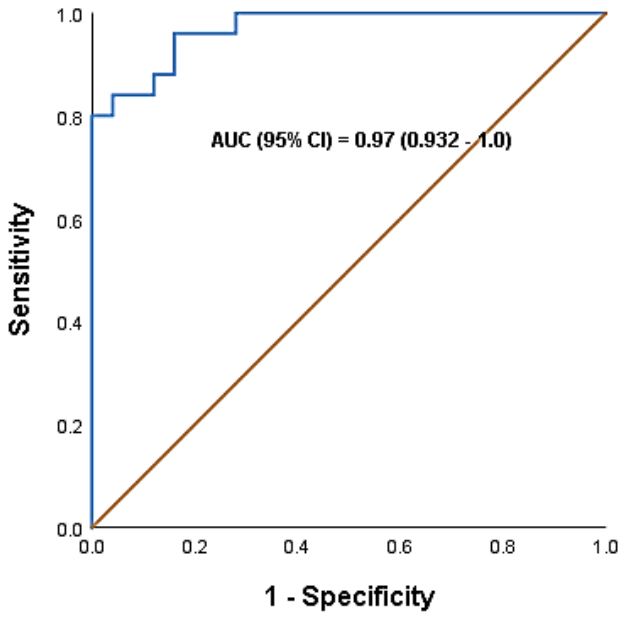


**Fig. (5).** ROC analysis of CCR6 in diagnosing MI.

**Table 1. Baseline clinical and demographic characteristics in both groups.**

| - | - | **Group I AMI (n = 25)** | **Group II Controls (n = 25)** | ***P*-value** |
|---|---|---|---|---|
| Age (years) | Mean ± SD | 54 ± 9 | 52 ± 10 | 0.542 |
| Gender n (%) | Males | 15 (60.0) | 14 (56.0) | 0.774 |
| | Females | 10 (40.0) | 11 (44.0) | |
| Smoking | n (%) | 9 (36.0) | 9 (36.0) | 1.0 |
| Diabetes mellitus | n (%) | 10 (40.0) | 11 (44.0) | 0.774 |
| Hypertension | n (%) | 17 (68.0) | 15 (60.0) | 0.556 |
| Body mass index | Mean ± SD | 26.15 ± 2.16 | 25.26 ± 2.16 | 0.153 |

**Note:** Independent t-test was used for numerical data. Chi-square test was used for categorical data. AMI= Acute myocardial infarction.

**Table 2 Hemodynamic parameters and Gensini score in both groups.**

| - | - | **Group I AMI (n = 25)** | **Group II Controls (n = 25)** | ***P*-value** |
|---|---|---|---|---|
| Heart rate (b/m) | Mean ± SD | 73 ± 2 | 73 ± 2 | 0.897 |
| Respiratory rate (c/m) | Mean ± SD | 16 ± 1 | 15 ± 1 | 0.909 |
| Temperature (°C) | Mean ± SD | 36.9 ± 0.4 | 37 ± 0.3 | 0.688 |
| Systolic blood pressure (mmHg) | Mean ± SD | 125 ± 6 | 126 ± 5 | 0.517 |
| Diastolic blood pressure (mmHg) | Mean ± SD | 84 ± 4 | 86 ± 4 | 0.155 |
| Gensini score | Mean ± SD | 59.4 ± 24 | - | - |

**Note:** Independent t-test was used. AMI= Acute myocardial infarction.

**Table 3. Complete blood count components with differential counts in both groups.**

| - | - | **Group I AMI (n = 25)** | **Group II Controls (n = 25)** | ***P*-value** |
|---|---|---|---|---|
| TLC ($\times 10^3/\mu l$) | Mean ± SD | 8.4 ± 2.55 | 7.82 ± 2.02 | 0.377 |
| Lymphocyte (%) | Mean ± SD | 29.7 ± 8.1 | 31.6 ± 7.7 | 0.409 |
| Monocyte (%) | Mean ± SD | 5.8 ± 2 | 5.1 ±1.5 | 0.160 |
| Granulocyte (%) | Mean ± SD | 61.9 ± 9.4 | 60.9 ± 7.3 | 0.686 |
| RBCs ($\times 10^6/\mu l$) | Mean ± SD | 4.75 ± 0.56 | 4.71 ± 0.48 | 0.793 |
| Hemoglobin (g/dl) | Mean ± SD | 12.9 ± 1.3 | 13.1 ± 1.8 | 0.539 |
| Hematocrit (%) | Mean ± SD | 41 ± 4.6 | 42.3 ± 4.3 | 0.328 |
| MCV (fl) | Mean ± SD | 87.11 ± 7.02 | 88.18 ± 4.21 | 0.519 |
| MCH (pg) | Mean ± SD | 27.9 ± 1.53 | 27.98 ± 1.66 | 0.852 |
| MCHC (g/dl) | Mean ± SD | 33.35 ± 1.93 | 32.63 ± 2.55 | 0.269 |
| Platelets ($\times 10^3/\mu l$) | Mean ± SD | 273 ± 55 | 273 ± 64 | 0.992 |

**Note:** Independent t-test was used. AMI= Acute myocardial infarction.

**Table 4. Lipid profile findings in both groups.**

| - | - | **Group I AMI (n = 25)** | **Group II Controls (n = 25)** | ***P*-value** |
|---|---|---|---|---|
| Total cholesterol (mg/dl) | Mean ± SD | 239 ± 45 | 221 ± 35 | 0.120 |
| Triglycerides (mg/dl) | Mean ± SD | 158 ± 28 | 161 ± 18 | 0.683 |
| LDLc (mg/dl) | Mean ± SD | 161.6 ± 44.4 | 141.5 ± 43.3 | 0.112 |
| HDLc (mg/dl) | Mean ± SD | 44 ± 10 | 50 ± 11 | 0.029 |

**Note:** Independent t-test was used. LDL; Low-density lipoprotein, HDL; High-density lipoprotein. AMI= Acute myocardial infarction.

**Table 5. Serum uric acid and Chemokine receptor 6 (CCR6) in both groups.**

| - | - | **Group I AMI (n = 25)** | **Group II Controls (n = 25)** | ***P*-value** |
|---|---|---|---|---|
| Serum uric acid (mg/dl) | Mean ± SD | 6.2 ± 0.9 | 5.5 ± 1.1 | 0.018 |
| CCR6 (%) | Mean ± SD | 32.9 ± 8.0 | 16.6 ± 6.7 | <0.001 |

**Note:** Independent t-test was used. AMI= Acute myocardial infarction.

**Table 6. Multivariate logistic regression analysis in prediction of MI.**

| - | **OR (95% CI)*** | ***P*-value** |
|---|---|---|
| CCR6 % | 1.831 (1.176 – 2.851) | 0.007 |
| Serum uric acid (mg/dl) | 2.268 (1.113-4.539) | 0.021 |
| HDLc (mg/dl) | 0.929 (0.868 – 0.994) | 0.033 |

**Note:** *Adjusted for age, gender, DM, HTN, and smoking. OR: Odds ratio, 95% CI; 95% confidence interval. AMI= Acute myocardial infarction.

Epidemiological studies show that uric acid is a poor predictive indicator for death in those with previous heart failure and an independent predictor for cardiovascular adverse events [22].

It is imperative to mention here that other molecules may be involved in the genesis of these events such as natural anticoagulants [23, 24]. Some other studies also have suggested that possible contributing factors in the development of such thrombotic events are microparticle generation and adhesion molecules expressed on white cells [25].

For the purpose of predicting MI, we used multivariate logistic regression analysis. It revealed that CCR6 expression levels on peripheral blood lymphocytes, serum uric acid, and HDLc were significant independent predictors for MI, adjusting for the impact of age, gender, hypertension, diabetes, and smoking. Additionally, in the AMI group, the proportion of CCR6+ lymphocytes was positively linked with the degree of coronary artery stenosis as assessed by the GS, indicating a critical function for CCR6 in the emergence of atherosclerosis. One of the important limitations of the current study is the small sample size, which rendered some statistical tests' results non-significant.

## CONCLUSION

The percentage of CCR6+ expression on lymphocytes is an independent biomarker for AMI. Additionally, T cells, which are implicated in the immunological response and connected to the instability of coronary lesions, are recruited by CCR6 as a mediator.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AMI | = | Acute Myocardial Infarction |
| AUC | = | Area Under Curve |
| BMI | = | Body Mass Index |
| CBC | = | Complete Blood Count |
| CCR | = | Chemokine Receptor |
| FITC | = | Fluorescein Isothiocyanate |
| GS | = | Gensini Score |
| HDLc | = | High-density Lipoprotein |
| HIV | = | Human Immunodeficiency Virus |
| iDCs | = | Immature Dendritic Cells |
| LAD | = | Left Anterior Descending Coronary Artery |
| LCX | = | Left Circumflex Artery |
| MPV | = | Mean Platelet Volume |
| NLR | = | Neutrophil-lymphocyte Ratio |
| PBS | = | Phosphate-buffered Saline |
| PDW | = | Platelet Distribution Width |
| PE | = | Phycoerythrin |
| TC | = | Total Cholesterol |
| TG | = | Triglycerides |

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

After receiving the MS 1-12-2018 permission number from the local Benha Faculty of Medicine ethics committee, the study was launched. Everything was completed in accordance with the committee's instructions. Before collecting any samples, everyone gave their voluntary informed consent.

## HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All procedures performed in studies involving human participants were in accordance with the ethical standards of institutional and/or research committee and with the 1975 Declaration of Helsinki, as revised in 2013.

## CONSENT FOR PUBLICATION

Informed consent was obtained from all participants.

## STANDARDS FOR REPORTING

The methodology and STROBE principles were followed.

## AVAILABILITY OF DATA AND MATERIALS

The data that support the results and findings of this research are available from the corresponding author [AMB], upon reasonable request.

## FUNDING

None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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