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Research Article

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Investigation of TLR4, TLR6, TLR7, and CD36 Expression on T lymphocytes in Coronary Artery Disease

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Abstract

Background: Coronary artery disease (CAD) is among the significant causes of death globally, caused by fatty deposits in blood vessel walls. Increasing evidence indicates that toll-like receptors (TLRs) are pivotal to atherosclerosis progression. The function of CD36 as a glycoprotein in atherosclerosis was also suggested. This study aimed to investigate the levels of TLR4, TLR6, and TLR7, as well as CD36 cell surface markers in CAD. **Methods:** This study included 64 patients undergoing angiography to determine whether atherosclerosis was present. The patients were categorized into two groups. The three main coronary arteries are all stenosed at over 50% in patients with CAD^{+} (n = 22) as cases and CAD^{-} (n = 42) with smooth angiography as controls. Each patient's syntax score (SS) and Gensini (vessel score) were calculated. The TLR-4, TLR-6, TLR-7, and CD36 expression were measured using Flow cytometry. **Results:** TLR4 and TLR6 showed a significant link with the Gensini score in the subject group. In addition, TLR7 had an association with Syntax scoring in this study. **Conclusions:** The findings illustrated the association between TLR4, TLR6, and TLR7 cell surface markers with Gensini and Syntax scoring in individuals with coronary artery disease.

Keywords:
coronary artery disease; TLR4; TLR6; TLR7; CD36

1. Introduction

Coronary artery disease (CAD) is the leading cause of death worldwide, primarily due to atherosclerosis [1,2]. Several reasons are involved in atherosclerosis pathogenesis, which occurs when lipids accumulate on artery walls, including genetic and environmental factors [3,4]. Women older than 55 and men older than 45, smoking, family background, diabetes, high blood pressure, high body mass index (BMI) as well as a sedentary lifestyle, declined level of High-Density Lipoprotein (HDL), raised level of Low-Density Lipoprotein (LDL), and cholesterol are the most prominent risk factors of CAD as a chronic disease [5,6]. The World Health Organization (WHO) reported that 60% of the economic burden of CAD is in developing countries [7]. CAD is responsible for 7.2 million deaths per year, which is equal to 12% of death rates annually [7]. The cost of treating cardiovascular disease is $315.4 million in America [8,9]. The role of family background and the 50% heredity rate in the development

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of CAD has been proposed by genetic studies [10,11]. Immune system cells and inflammation form vascular plaque and cardiovascular disease [12,13].

CAD can be prevented, controlled, and treated by further study of immune cells and other inflammatory factors. TLRs are receptors on the cell membrane that are important for immunity and respond to molecules from pathogens [14,15]. They are also found in the cells that present antigens (macrophages and dendrites), which protect against infections in the first line [16]. It could further be understood that TLRs trigger intracellular signaling pathways by cytokines. Studies show a link between TLRs, which start the inflammatory response, and atherosclerosis, an inflammation-related disease [17,18]. Moreover, TLR4 is one of the TLRs highly expressed in heart cells [19,20]. This also signals inflammation through the interleukin receptor and is related to high blood pressure and heart muscle damage [19,20]. Physical exercise can help prevent heart diseases by lowering the TLR4 expression [21]. TLR-6 is another type of TLR that works with TLR2 and CD36 to activate cells [22,23]. Eliminating the TLR6 gene reduced the risk of heart muscle scarring [24]. TLR6 also helps control oxidative stress and inflammation-related substances like IL-6 and cytokines using the NF-κB signaling pathway [24]. Macrophages in atherosclerotic plaques produce TLR7 [25]. When the TLR7 gene is missing or low, it leads to atherosclerosis and inflammation in the blood vessels [26]. Having said this, more TLR7 in the plaques is linked to improved conditions and lower risks of heart problems in patients [26,27].

CD36 is a glycoprotein on fat cells and macrophages that helps balance lipids and immune responses in inflammation [28,29].

The expression of the CD36 gene is linked to persistent inflammation and immune responses that heighten the risk of atherosclerosis. Consequently, the deletion of this gene reduces the likelihood of atherosclerotic lesion formation [28,29]. The SRC proto-oncogene, non-receptor tyrosine kinase (Src) family facilitates the communication between CD36 and TLRs. CD36 triggers the phosphorylation and activation of TLR4 and TLR6 via Lyn kinase [29]. CD36 ligands such as ox-LDL trigger the creation of (CD36-TLR4-TLR6) heterodimer and NF-κB activation [30]. Angiography is a treatment procedure used to diagnose vascular occlusion among individuals suffering from cardiovascular disorders. Gensini's score is employed to evaluate the severity of coronary artery disease (CAD) in individuals by assessing stenosis grade [31].

The Syntax score also evaluates coronary artery lesions stenosis. A higher Syntax score means the stenosis is more severe [32]. In this research, we investigated the levels of TLR4, TLR6, TLR7, and CD36 cell surface markers and their correlation with Genisini and Syntax scores in coronary angiography patients.

2. Materials and Methods

2.1. Patient Enrollment

This research comprises patients who underwent diagnostic coronary angiography at Dr. Shariati Hospital from 2019 to 2020. There were two groups of participants, CAD+ and CAD-. The CAD+ group had 22 patients with over 50% narrowing in the three main coronary arteries (right coronary artery, left anterior descending artery, and left circumflex coronary artery).

The CAD group consisted of 42 patients with normal angiography results. Prior to performing the angiography, the researchers obtained written consent and collected relevant medical records from the patients. After the procedure, the angiography data were saved on a CD and analyzed by an intervention cardiologist. The Syntax and Gensini (vessel scores) were analyzed for each patient according to the American Heart Association (AHA) guidelines. The ethics committee of the Endocrinology and Metabolism Research Institute at Tehran University of Medical Sciences approved this study.

With regard to the exclusion criteria, this study did not include patients with congenital heart valve defects, familial hypercholesterolemia, or a history of Percutaneous Coronary Intervention (PCI) and Coronary artery bypass grafting (CABG). The selection criteria were based on stenosis in at least 70% of the arterial cross-section of all three coronary arteries, which was confirmed by angiography. Participants in the control group exhibited no signs of stenosis, as confirmed by angiography. Patients who had taken hypertension medication before or had a blood pressure reaching or exceeding 140/90 mm Hg were classified as having hypertension. Diabetes mellitus, along with hyperlipidemia, was detected using the American Diabetes Association (ADA) criteria. Significant diameter stenosis >70% was also considered when calculating the Gensini (vessel score). An American Heart Association grading system was also applied to evaluate the syntax score [33].

2.2. Sample Collection and Processing

To identify cell surface markers, the researchers collected 10 mL of blood in EDTA tubes and stained it with a DAKO uti-lyse erythrocyte lysing reagent kit. They then used the Flow Cytometry method to investigate the expression

2024, Vol. 1, Article ID. 2024.0004
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of TLR4, TLR6, TLR7, and CD36 markers in CD3+ T-lymphocytes.

2.3. Statistical Analysis

The analyses were conducted statistically using version 16 of SPSS software. A natural logarithm (LN) was applied to normalize cell surface markers distribution. An unpaired *t*-test and a chi-square test were used to analyze the statistics. Pearson correlation was also used to examine the relationship between the scoring system and cell-surface markers. The correlation between cell surface markers and Gensini and Syntax scores was calculated using linear regression analysis. Statistics indicated that a *p*-value lower than 0.05 was significant.

3. Results

While comparing CD36, TLR4, TLR6, and TLR7 expression between the two groups, there was no statistical significance in the difference for TLR4 ($p = 0.24$) (Figure 1), TLR6 ($p = 0.29$) (Figure 2), TLR7 ($p = 0.38$) (Figure 3), and CD36 ($p = 0.22$) (Figure 4).

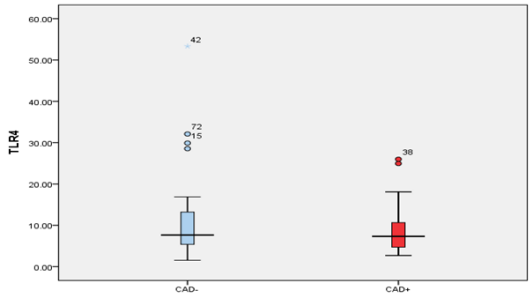

Figure 1: TLR-4 cell surface marker. The TLR4 expression was higher in CAD$^+$ patients (34.55 ± 1.75) compared to CAD$^-$ (29 ± 0.67). However, statistically, there was no significance in the difference ($p = 0.24$).

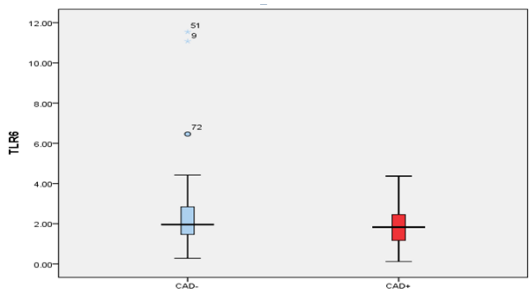

Figure 2: TLR-6 cell surface marker. The expression of the TLR6 cell surface marker was lower in CAD+ (27.80 ± 14.52) compared to CAD- (32.81 ± 2.34), but this also did not reach the statistically significant level ($p = 0.29$).

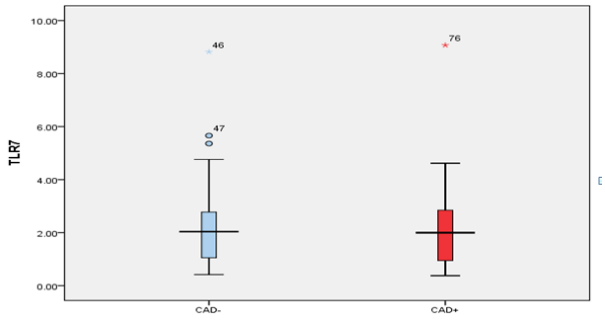

Figure 3: TLR7 cell surface marker. The comparison of TLR7 cell markers was not significantly different between CAD$^+$ (33.66 ± 15.33) and CAD$^-$ (29.50 ± 1.78) groups ($p = 0.38$).

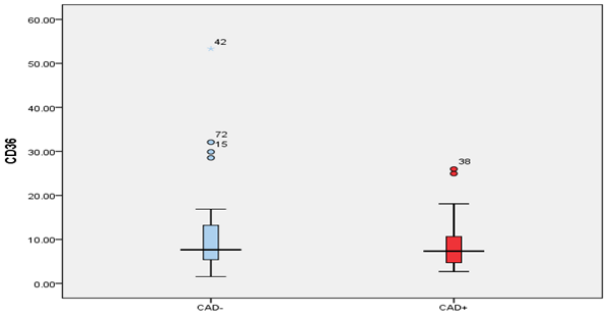

Figure 4: CD36 cell surface marker. The expression of the CD36 cell surface marker in CAD+ (27.34 ± 11.62) was lower than in CAD- (33.06 ± 10.08), but this difference was not statistically significant ($p = 0.22$).

4. Correlation of Markers Expression with Gensini Score and Syntax score

Table 1 presents the clinical traits of the participants. The prevalence of diabetes mellitus was considerably higher among the CAD+ (56.3%) than among the CAD- (43.8%) ($p = 0.03$). However, there were no notable variations between the two groups regarding age ($p = 0.76$), BMI ($p = 0.41$), gender ($p = 0.57$), smoking history ($p = 0.45$), hypertension ($p = 0.78$), in addition to hyperlipidemia ($p = 0.06$).

Table 2 illustrates Pearson results test correlated between cell surface markers expression and the Gensini and Syntax scoring system. The Gensini score exhibited a positive correlation with TLR4 ($p = 0.01$) and TLR6 ($p = 0.03$) but not with TLR7 ($p = 0.39$) or CD36 ($p = 0.15$). There has not been any significant link between TLR4 ($p = 0.54$), TLR6 ($p = 0.43$), CD36 ($p = 0.85$), and the Syntax score. However, TLR7 showed a notable link with the Syntax score ($p = 0.01$).

Furthermore, linear regression was applied as an independent factor to assess the correlation between Gensini and Syntax scores and cell surface markers as a dependent factor. The results showed that TLR4 had a positive correlation with the Gensini score ($r^2 = 0.08$, $\beta = 0.29$, $p = 0.01$), while TLR6 had a negative correlation with the Gensini score ($r^2 = 0.06$, $\beta = -0.26$, $p = 0.03$). However, the correlation between TLR7 ($r^2 = 0.01$, $\beta = -0.10$, $p = 0.39$) and CD36 ($r^2 = 0.03$, $\beta = 0.17$, $p = 0.15$) with Gensini score of no statistical significance in difference has been shown. Similarly, it was deduced that there was no considerable correlation between TLR4 ($r^2 = 0.01$, $\beta = -0.11$, $p = 0.54$), TLR6 ($r^2 = 0.02$, $\beta = 0.14$, $p = 0.43$), and CD36 ($r^2 = 0.001$, $\beta = 0.03$, $p = 0.85$) and Syntax score. However, TLR7 was negatively correlated with the Syntax score ($r^2 = 0.18$, $\beta = -0.43$, $p = 0.01$).

5. Discussion

Although the incidence of CAD is decreasing in industrialized nations, it is on the rise in the underdeveloped nations. Additionally, CAD is responsible for 80% of deaths in developing countries [34,35]. TLRs belong to the interleukin-1 receptor/family of toll-like receptors also expressed on the surface of mammalian cells. Thirteen TLRs have been identified so far [36]. More recently, a study conducted as the CANTOS phase 3 trial has shown that direct reduction of inflammation with a monoclonal antibody treatment (canakinumab) which is an anti-interleukin (IL)-1β antibody, decrease cardiovascular event rates. Further, the results approve inflammation as a remarkable target in cardiovascular disease prevention [37].

This research has checked the link between CAD and three members of the TLR family, namely TLR4, TLR6, TLR7, and CD36. These receptors are involved in inflammation and atherosclerosis [28]. According to this study, diabetes presence is more significant among CAD+ individuals than CAD- individuals ($p < 0.05$). Additionally, it has been demonstrated that there is a correlation between diabetes and CAD, as individuals with diabetes are more susceptible to developing CAD [38].

Cardiovascular disease is associated with TLR4 through cytokines and immune responses [39]. TLR4 expression has been detected in different cells, including cardiomyocytes, macrophages, endothelial cells, and smooth muscle cells [40]. This study found that TLR4 levels were elevated among CAD+ cases, which contrasted with the CAD- cases, although there was no statistical significance in the difference ($p > 0.05$). Similar to the findings, high levels of TLR4 expression were found in atherosclerotic plaques in both rats and humans. However, this gene was not expressed in normal vessels [17,41–43]. Moreover, the findings revealed a positive correlation between TLR4 and the Gensini score, suggesting that TLR4 might contribute to an increased risk of coronary stenosis ($p < 0.05$). Yet, no association has been found between TLR4 and Syntax score in the population studied ($p > 0.05$). The roles of TLR4 and ApoE in atherosclerosis were investigated in mice deficient in TLR4 and ApoE [44]. Contrary to our study, it was discovered that mice lacking both ApoE and TLR4 (ApoE−/−TLR4−/−) were more susceptible to atherosclerosis and inflammation, and they exhibited an increase in lesion formation. This indicates that TLR4 may protect against atherosclerosis [44]. The Asp299Gly polymorphism of the TLR4 gene, known to reduce the inflammatory response, has been associated with atherosclerosis risk [40]. Furthermore, Chlamydia pneumonia has been involved in the proliferation of smooth vascular muscle cells via TLR4 [45,46].

According to our study, the CAD- group exhibited a higher level of TLR6 cell marker than the CAD+ group ($p > 0.05$), and TLR6 appeared to have a protective influence in opposition to coronary artery disease, as it negatively impacted coronary stenosis (Gensini score) ($p < 0.05$). However, no association was found between TLR6 and the Syntax score ($p > 0.05$). The Pro249Ser polymorphism (rs5743810) of the TLR6 gene has been previously confirmed to be associated with atherosclerosis [47]. TLR6 has been linked to myocardial fibrosis through inflammatory pathways and oxidative stress [24]. In ApoE−/− deficient mice, TLR7 has been demonstrated to control the inflammatory response and inhibit atherosclerotic lesion progression in the CAD [48].

While the CAD+ group had higher TLR7 expression than the CAD- group, this difference had no statistical significance ($p > 0.05$). Although TLR7 did not significantly influence the Gensini score, it had a notable negative impact on the Syntax score, suggesting its involvement in the development of coronary artery lesions ($p < 0.05$). Macrophages lacking CD36 exhibited reduced pro-inflammatory properties, suggesting CD36 plays a pro-atherogenic role [49]. Given LDL's role in atherosclerosis, CD36 is responsible for 50% of LDL binding activity, indicating its association with cardiovascular disease [50]. CD36 has also been correlated with the cardiovascular disorder progression in diabetes [51]. In mice lacking CD36 and apoE (CD36-/apoE-), a 61% reduction in lesion area in females and a 74% reduction in males were observed.

However, in this research, CD36 expression was lower in the CAD+ cases than in the CAD- cases ($p > 0.05$), and CD36 had no correlation or effect on the Gensini

2024, Vol. 1, Article ID. 2024.0004
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Table 1: Clinical characteristics of patients.

Qualitative Variables	CAD^{+} (n = 22)	CAD^{-} (n = 42)	*p*-Value
Age	56.40 ±7.33	55.85 ± 6.87	0.76
BMI [a]	26.81 ± 3.22	27.69 ± 4.33	0.41
Gender (Female)	4(28.6%)	10(71.4%)	0.57
Gender [12]	18(36.7%)	31(63.3%)	
History of smoking	5(27.8%)	13(72.2%)	0.45
Diabetes Mellitus	9(56.3%)	7(43.8%)	0.03 *
Hypertension	11(33.3%)	22(66.7%)	0.78
Hyperlipidemia	9(52.9%)	8(47.1%)	0.06

[a]: BMI: Body Mass Index; * *p*-Values < 0.05 was regarded as significant.

Table 2: Correlation of Cell surface markers with Gensini and Syntax scoring system.

Gensini Score	TLR4	TLR6	TLR7	CD36
r^2 [a]	0.08	0.06	0.01	0.03
Beta-coefficient	0.29	−0.26	−0.10	0.17
p-value	0.01 *	0.03 *	0.39	0.15
Syntax score				
r^2	0.01	0.02	0.18	0.001
Beta-coefficient	−0.11	0.14	−0.43	0.03
p-value	0.54	0.43	0.01 *	0.85

[a]: r^2; * *p*-Values < 0.05 was deemed to be statistically meaningful.

and Syntax scores ($p > 0.05$). Soluble CD36 concentration has been illustrated as protective against metabolic syndrome progression in individuals with CAD [52]. The CD36 gene variants rs3173798 and rs3211892 showed a correlation with myocardial infarction [53]. CD36 mRNA expression was considerably higher in individuals with coronary heart disease (CHD) than in the control group [50].

6. Conclusions

Although the study has several limitations including small samples size which may be the cause of lack of significant differences in the expression of the studied cell surface markers between the CAD+ and CAD- groups, a notable correlation was found between the Gensini and Syntax scoring systems and TLR4, TLR6, and TLR7. Accordingly, it has been deduced that cell surface marker expression is associated with stenosis in patients with CAD. As such, further investigation into the relationship between cell surface markers and CAD in future studies with larger sample sizes could be beneficial. If these results are confirmed in future research, it could aid in identifying more effective strategies for preventing and managing CAD, considering thatthis disease imposes a substantial economic burden on societies.

List of Abbreviations

CAD	Coronary Artery Disease
TLRs	Toll-like Receptors
LDL	Low-Density Lipoprotein
NF-κB	Nuclear Factor- Kappa B Subunit

Author Contributions

S.A.: Performing lab experiments and Writing—Original Draft; E.S.: Writing—Original Draft; E.E.G.: Writing—Original Draft; S.P.-D.: Performing lab experiments and Writing—Original Draft; S.K.H.: sample collection; F.E.: Writing—Review & revision; M.M.A.: Conceptualization and supervision.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article and its supplemen-tary information files.

Ethics Committee Approval and Consent to Participate

The study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran, with the identification code 1744-106-03-1392. All patients involved in the study signed a written informed consent form.

Human Rights Statement

The research has been conducted according to the Declaration of Helsinki.

Consent for Publication

Not applicable.

Conflict of Interest

The authors declare no conflicts of interest.

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