GenoMed Connect
ISSN: 3006-9033
2024, Article ID. 2024.0006, Cite as: www.doi.org/10.69709/GenomC.2024.116041

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Research Article

OPEN ACCESS

Evaluation of IL 12 Gene Expression and Serum Levels of OxPL / apoB in Patients with Coronary Artery Disease

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Article History
Submitted: May 18, 2024 Accepted: October 20, 2024 Published: November 06, 2024

Abstract
Background Atherosclerotic lesions are formed as a result of low-density lipoprotein (LDL) accumulation, which is produced by oxidizing enzymes and oxidized phospholipids (OxPL). Phospholipid oxide causes inflammation-inducing activation genes and hyper-inflammation in addition to the initiation of inflammation and expression of Th1 cytokines. Interleukin 12 (IL-12) is one of the most significant stimulants of inflammation and immune responses, among the Th1cytokines. Therefore, it may play a significant role in contributing to atherosclerosis. The quantitative expression of IL-12 mRNA, along with serum OxPL levels, has been analyzed in patients with coronary artery disease (CAD). Methods: Coronary angiography patients, aged 45 to 65 years and referred with chest pain, were examined. The patients were classified into normal coronary arteries (N = 38) and severe three-vessel involvement coronary arteries (N = 41). Molecular testing was performed using real-time PCR. Also, an ELISA test was used to check the OxPl level. Results Expression of IL12 in Individuals with coronary artery disease was increased but was not statistically significant.

Keywords:
coronary artery disease; atherosclerosis; cytokine; OxPL; IL12

1. Introduction

Inflammation, has been known a major contributing factor to coronary artery disease (CAD) [1]. Atherosclerosis, which is an inflammatory illness affecting the walls of large- and medium-sized arteries, results from excessive low- density lipoprotein (LDL) cholesterol in the bloodstream [1,2]. Adaptive immune responses have a crucial function in this disease and atherosclerotic lesions are mostly formed by macrophages and T lymphocytes [3,4]. Generally, plaque T cells predominantly are made by helper T cell type 1(Th1), which secretes signaling proteins including interferon (IFN), IL-12, as well as tumor necrosis factor [5–8], which participate in macrophage activation and inflammation [9]. A study by Fernandez et al. illustrated that in plaque formation and rupture, there was a significant increase in serum IL-12 and intracellular expression of IFN-γ in CAD patients [4]. However, the association between these markers and atherosclerosis development is not fully understood [10].

It is known that IL-12 has a crucial function in regulating Th1 cellsThis heterodimer consists of two components: P35 (small unit) and P40 (heavy unit) [11]. It is produced by cells with phagocytic activity, as well as by genes responsible for antigen presentation, when stimulated by various microorganisms and their products [12–14]. In addition

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to its importance for Th1 differentiation, it stimulates natural killer cell proliferation and differentiation [15]. Studies have shown that in animal models, IL-12 acts as a pro-atherogenic molecule, due to the smaller size of plaques built up in arteries in IL 12 -/-/apoE -/- mice than in the control group [10–17]. IL-12 is an important factor that regulates immune response during early stage of atherosclerosis in apoE-/- mice [16]. The Important roles of IL-12 include the inflammatory process as well as the cellular immune response. Previous study revealed that interleukin-12 and interleukin-10 (two important cytokines that impact T lymphocyte differentiation into Th1 and Th2 phenotypes.) are expressed in macrophages of atherosclerotic lesions. Immunohistochemical studies have shown that human atherosclerotic plaques contain higher levels of IL-12 p70 compared to normal arteries [5]. The oxidation of LDL is an initial occurrence within the sub-endothelial space, rendering it susceptible to the development of atherosclerotic lesions. Subsequently, macrophages uptake these particles through scavenger receptors, leading to the formation of lipid-laden cells [10,17]. Atherosclerosis is identified by the accumulation of low-density lipoprotein within the arterial walls, which attracts immune cells and induces severe inflammation. Further, apolipoprotein B100 (ApoB100) is an important protein moiety of LDL that can cause an autoimmune response [8]. ApoB100 is characterized by the LDL exchangeable protein element, and atherosclerosis development is correlated with immunological reactions to peptides derived from ApoB100 (Discovery of a risk-associated peptide derived from apolipoprotein B100 (ApoB DS1) causing inherent responses which contribute to atherosclerosis) [18]. In particular, oxidized phospholipids associated with apolipoprotein B-100 particles (OxPL/apoB) leads to the progression of femora, carotid and coronary artery disease [8,19]. Studies have indicated an increase in OxPL/apoB levels in individuals with percutaneous coronary intervention [20–22]. The predictive efficacy of OxPL/apoB is enhanced by factors like Lp(a) and enzymes such as lipoprotein-associated phospholipase A2 and secretory phospholipase A2, which are considered as therapeutic targets [23].

Insight into the involvement of lipoprotein oxidation in CAD could enable more logical and precisely targeted diagnostic and therapeutic approaches in clinical applications. The oxidation of lipoproteins, particularly low-density lipoproteins, is a critical, if not essential, process for the formation of macrophage-derived foam cells, which are a key factor in the initiation of atherosclerotic plaques [2].

This study aimed to determine IL12 expression in unstimulated peripheral blood lymphocytes (PBMCs) from patients CAD. A comparison was made between this cytokine profile in unstimulated peripheral blood mononuclear cells (PBMCs) of individuals with and without CAD. Furthermore, we measured serum levels of apoB and OXPL in patients with coronary artery disease.

2. Methods

2.1. Subjects

A case-control association study has been conducted. The study received approval from the Ethics Committee of Tehran University of Medical Sciences (TUMS). All subjects and patients signed written informed consent before participating in this study. A personal/demographic questionnaire was completed, also. Patients who underwent coronary artery angiography due to chest pain at the Cath Lab Center of Dr. Shariati Hospital between 2010 to 2011 in Tehran, Iran were recruited to the study. Further, a trained cardiologist conducted and interpreted the procedures.

The study comprised of 38 consecutive individuals diagnosed with coronary artery disease (CAD+) based on coronary angiography, displaying significant arterial stenosis. Furthermore, 41 consecutive individuals with normal coronary angiographic studies (considered to be CAD-) were participated in this investigation.

Patients with underlying cardiovascular disease, were excluded e.g., congenital heart valve defects, and familial hypercholesterolemia. CAD+ was considered if severe stenosis, which is characterized as a decline in internal diameter of more than 50% in all three main coronary arteries were present. Patients without having this criterion were considered as CADHigh blood pressure, diabetes, hyperlipidemia, smoking status, and a family history of other cardiovascular diseases, including myocardial infarction and premature coronary heart disease in first-degree relatives, were documented for all participants. In this study, hypertension was diagnosed in subjects who had taken blood pressure medications or had an average blood pressure of $\geq$140/90 mmHg. Diabetic mellitus and dyslipidemia were detected based on the American Diabetes Association [23] criteria and National Cholesterol Education Program adult treatment panel III, respectively. The study received approval from the Ethics Committee of Tehran University of Medical Sciences (TUMS).

2.2. RNA Isolation and cDNA Synthesis

A total of 6 cc of peripheral blood was collected from each participant in tubes containing heparin, and RNA was extracted from the cellular RNA as described in Tripure iso-

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lation reagents (Roche) manufacturer instructions. In this method, 100 µL of chloroform was added to PBMC and then vortexed for 15 s. The sample was placed away from the light for 3 min. In the next step, the centrifuge was performed with a speed of 12,000 g and 4 °C for 15 min. The colorless phase was carefully transferred to a 1.5 mL tube and the same volume of isopropanol was added. After inverting several times, it was placed at −20 °C for 10 min and then centrifuged at 12,000 g at 4 °C for 10 min. After draining the supernatant, one ml ethanol (75%) was added to it and vortexed for a few seconds, afterward the centrifuge was performed for 10 min at 12,000 g at 4 °C. Finally, ethanol was discarded, and RNA pellets were dissolved the in 30 µL of DEPC water and stored at −80 °C for storage. On a Nano-Drop spectrophotometer (Nano-Drop Thermo Scientific 2000), we measured the OD 260/280 ratio and OD 260 to determine the purity and concentration of RNA. After that, we discarded preparations with an OD 260/280 ratio lower than 1.8 and used the Revert Aid First Strand cDNA Synthesis Kit, lot no. K1622 (Thermo Fisher Scientific Company) for cDNA synthesis.

2.3. Real-Time PCR

In this study, real-time PCR was conducted using SYBR Green I (Takara, Japan) and an ABI StepOne™ real-time PCR device (Applied Biosystems, CA, USA) to measure cytokine mRNA expression levels.48-well plates were used to conduct the reaction with a total volume of 20 µL consisting of 10 µL of 2 × SYBER premix EX Taq, 0.5 µL of forward and reverse primers (1 µM), 6 µL of deionized water and 3 µL of 10-fold diluted cDNA product. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) housekeeping gene was applied as an internal control for normalization. To amplify specific oligonucleotide primers, PCR reactions were conducted in duplicate. Table 1 lists the primer pair sequences. Real-time PCR for *IL12/HPRT* gene was carried out with a primary denaturation phase of 10 s at 95 °C, 40 cycles at 95 °C for 5 s, and 61 °C for the 40 s. We performed melt curve analysis at 95 °C for 15 s, 61 °C for 15 s, and 95 °C for 15 s in the final step.

Gene expression data were normalized against Hprt as reference gene. Data Analysis was performed using the 2-ΔΔCT method.

Serum levels of OXPL/apoB was measured with Sandwich ELISA using Biomedica Medizinprodukte, Austria, Cat.No: BI-20022.

2.4. Statistical Analysis

The data from gene expression were adjusted using HPRT as the benchmark gene. The Independent-Sample T-Test was employed to ascertain importance of the differences in gene expression among control and test groups. The Kolmogorov-Smirnov test was employed to check for normal distribution of variables. Furthermore, REST was used for data analysis in real-time PCR. The analysis was conducted using SPSS version 15, as well as a P-value equal to or less than 0.05 was deemed to demonstrate significant analysis.

3. Results

Table 2 shows the characteristics of subjects with and without CAD. Patients with CAD+ more frequently exhibited classic cardiovascular risk factors compared to individuals without CAD (CAD-). Table 2

3.1. The Comparison of IL12 Gene Expression Between Subjects Diagnosed with CAD (CAD+) and Those Devoid of the Condition (CAD-)

Figure 1 illustrates the expression of the IL12 gene in both CAD+ patients and individuals without CAD (CAD-). Analyzing PCR data at a quantitative level revealed that no notable variation was observed in *IL12* gene expression between the groups ($p = 0.67$).

Once classic risk factors were taken into account, it was found that there was no association between CAD and IL-12. This was found to be the case regardless of potential confounding factors such as diabetes, high blood pressure, smoking, age, sex, as well as body mass index, Table 3.

Figure 2 illustrates the relationship between hypertension and OXPL. The average hypertension ± standard deviation was found to be 1.30 ± 2.59 in the group with OXPL, and 0.21 ± 0.65 in the group without OXPL. The link between hypertension and OXPL was found to be significant in this study ($p = 0.02$). It could be suggested that the presence of OXPL, which is a result of atherosclerosis, leads to an elevation in blood pressure. In this research, the correlation coefficient between IL12 and OXPL using Pearson correlations ($p = 0.95$) and Spearman's rho correlation ($p = 0.49$) was examined. Nevertheless, no notable distinction was observed in this aspect ($p > 0.05$).

Table 1: Primer sequences for real-time PCR quantification.

Amplicon Size	Primer Pair Sequences		Gene
131bp	5- CCTGGCGTCGTGATTAGTGAT -3	HPRT F	HPRT
	5- AGACGTTCAGTCCTGTCCATAA -3	HPRT R	
118bp	5- GAATTAACCAAGAATGAGAGTT-3	*IL12* F	*IL-12*
	CATAAATACTAAGGCACAG-3-5	*IL12* R	

Table 2: Baseline characteristics in Iranian subjects with and without CAD.

Variable	CAD^+ (n = 38)	CAD^- (n = 41)	*p* Value
Age *	57±6	56±7	0.3
Sex (male) (%)	79%	76%	0.7
Sex (female) (%)	21%	24%	0.7
Current smokers N (%)	26%	32%	0.6
Diabetes mellitus N (%)	45%	17%	0.008
Hypertension N (%)	55%	54%	0.9

* Variables are described based mean ± standard deviation.

Table 3: Logistic regression analysis shows the Association of risk factors with IL-12 expression and CAD.

		p Value	OR	95% C.I.
Step 1 [a]	*IL12* Expression	0.394	1.076	0.91–1.27
	age	0.662	1.019	0.93–1.11
	sex	0.339	0.465	0.09–2.23
	Smoking	0.908	1.071	0.33–344
	diabetes	0.013	0.189	0.05–0.70
	Hypertension	0.718	0.814	0.26–2.47
	BMI *	0.099	0.884	0.76–1.02
	Constant	0.134	407.284	

* Body Mass Index.

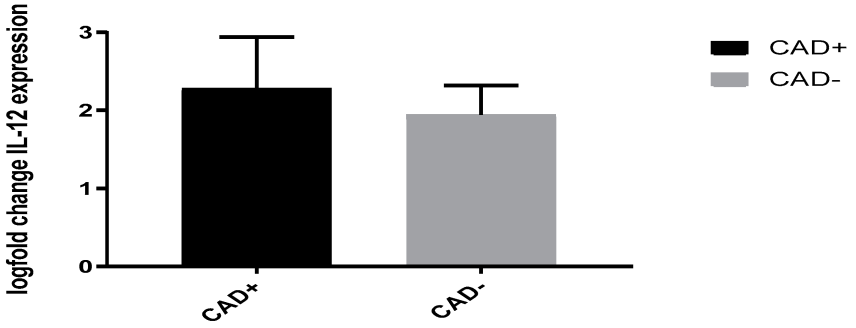

Figure 1: *IL12* gene expression in individuals with coronary artery disease as opposed to individuals without coronary artery disease. No notable distinction was seen in IL12 gene expression CAD+ versus CAD- ($p = 0.67$).

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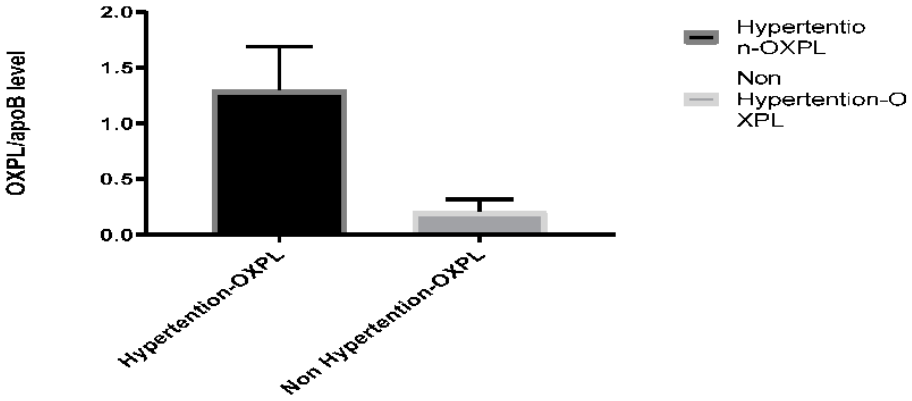

Figure 2: Association of hypertension with OXPL. A considerable distinction existed among hypertension as well as OXPL/apoB ($p = 0.02$).

4. Discussion

Coronary artery disease (CAD) is a major global contributor to both death and illness [24].

The development of this disease has been proposed to result from a disruption in the balance of fat metabolism and an abnormal immune response, which includes persistent inflammation of the arterial wall [24]. Several risk factors have been recognized in atherosclerosis progression, such as smoking, diabetes, high blood pressure, and irregular lipid concentrations [25]. The atherosclerotic lesion involves immune cells such as macrophages and T lymphocytes. In the progression of this disease, there is a chronic inflammation that affects major and average-sized arteries. This inflammation is triggered by buildup and oxidative modification of LDL, or "bad" cholesterol, within the arterial wall [1]. Primarily, inflammation enhancement is controlled by cytokine expression specific patterns [11], which has a crucial function in various stages of atherosclerosis, including the disruption of atherosclerotic plaque and thrombosis [26,27]. IL-12, a significant stimulator of Th1-type cytokines production, can therefore intensify atherosclerotic lesions [11]. IL-12 promotes a pro-atherosclerotic progression of Th1 cell phenotype, which is more prone to atherosclerosis [28,29]. Studies using immunohistochemistry have revealed an increased existence of IL-12 p70 in human atherosclerotic accumulations when in comparison with normal arteries [5]. It has been reported by Uyemura and colleagues that both IL-12 mRNAs and proteins were notably expressed in human atherosclerotic accumulations [5]. Research conducted by Lee and his team showed that giving IL-12 to mice lacking apolipoprotein-E could speed up the development of atherosclerosis in the aorta [10]. These results emphasize the significant role of IL-12 in atherosclerosis in experimental setups. In addition, Zhou and his team found that patients suffering from acute myocardial infarction had higher levels of IL-12 in their blood, implying that IL-12 might contribute to the onset of acute coronary syndrome [30]. Additional research indicates that the patterns of IL-12 expression are linked to atherosclerosis onset, also suggesting that IL-12 might hasten the progression of atherosclerosis [31]. These studies, which outline the role of IL-12 in promoting atherosclerosis, suggest that IL-12 could be a potential target for atherosclerosis treatment. The evidence is steadily growing that oxidized phospholipids (OxPLs) are significant contributors to atherosclerosis. These phospholipids are found in abundance in both human and mouse lesions. Previous reports have recognized role of certain OxPLs in controlling various cell types in vessel walls [32]. Numerous receptors and signaling pathways linked to the action of OxPL have been discovered and are found to be more active in human lesions. There is ample research that backs the role of OxPL/apoB levels as a predictive marker as well as an independent factor of risk for atherosclerosis. According to an angiography, the ratio of OxPL to apoB can independently forecast the coronary artery disorder existence [20]. In a research conducted on two different atherosclerosis animal models, OxPL ratio to apoB in plasma rose by a significant 50% to 100% [33].

In this research, an elevation in the IL12 gene expression in the unstimulated PBMCs of patients with CAD were observed. However, the results were not significant which might be due to a small sample size, involvement of other genetic factors, and that gene expression was examined in PBMCs rather than at the site of atherosclerotic lesions. In this study, other factors were also investigated that could influence atherosclerosis, and it was found that only blood pressure had a significant correlation with the OXPL level which confirms the previous finding [34].

Abbreviations

- OxPL: Oxidized phospholipids
- CAD: coronary artery disease
- LDL: low-density lipoprotein
- *Th1*: T-helper 1
- OxPL/apoB: oxidized phospholipids on apolipoprotein B-100 particles
- PBMCs: peripheral blood lymphocytes
- HPRT: Hypoxanthine-guanine phosphoribosyl transferase
- SD: standard deviation
- OxPLs: oxidized phospholipids
- OxLDL: oxidized low-density lipoprotein
- PC: phosphorylcholine

Author Contributions

S.P.-D.: Performing lab experiments and Writing—Original Draft; S.K.H.: sample collection; S.B.D.: Performing lab experiments and Writing—Original Draft; H.M.: Performing lab experiments and Writing—Original Draft; 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 supplementary information files.

Consent for Publication

Not applicable.

Conflict of Interest

The authors declare no conflicts of interest.

Funding

This project was supported for educational purposes by Endocrinology and Metabolism Research Institute, Tehran University of Medical Sciences under the grant number 1860-99-03-1393.

Acknowledgments

The authors appreciate Tehran University of Medical Sciences for their contributions.

Ethics Approval and Consent to Participate

The study was approved by ethical community of Tehran University of Medical. All patients included signed a written informed consent form.

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