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RESEARCH PAPER

Mutations in the Interleukin-15 Gene as a Molecular Biomarker in the Atherosclerosis Disease.

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A B S T R A C T:

The present study investigated the association between mutations in the gene of interleukin (IL)-15 located on exon 8 and the risk of atherosclerosis in Iraqi patients. A total of 87 patients (71 males and 16 females) with atherosclerosis prior to [Coronary Artery Bypass Graft (CABG) surgery] median age, 57 years and 30 subjects with no CVD (median age, 58.5 years) were enrolled at the Surgical Specialty Hospital of Cardiac Center-Erbil-Iraq between April 2021 and February 2022. Genotype analysis was achieved using a polymerase chain reaction (PCR) and Sanger DNA sequencing and clinical biochemistry were achieved by Cobas 311and e411 analyzers. The IL-15 homozygous rs2291596 (53.3%) and rs10833 (100%) genotypes appeared in peak frequencies and were related to a risk of the progress of atherosclerosis. In comparison, the remaining two novel mutations exhibited a low frequency for the 97264 G>GC genotype (13.3%) and the 97270 G>GT genotype (26.7%). Significant changes were observed in serum C-reactive protein (CRP) levels, the erythrocyte sedimentation rate (ESR), and high-density lipoprotein (HDL), alkaline phosphatase (ALP), blood fasting glucose, hemoglobin A1c (HbA1c) and troponin T-hs levels in the patients with atherosclerosis compared with those of the non-CVD subjects. Finally, it was concluded that the IL-15 mutations may play an essential role in the development and prognostic prediction of blood vessel atherosclerosis.

KEY WORDS: Atherosclerosis; C-reactive protein; Hemoglobin A1c; Interleukin-15; Troponin T-hs.
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1.INTRODUCTION :

Interleukin (IL)-15 is a cytokine that is involved in the inflammatory process with a structural analogy to IL-2; both bind to IL-2Rβ Clusters of Differentiation 122 (CD122) and IL-2Rγ (CD132) receptors and the main function of both ILs is present by the corresponding α receptor chains, IL-15Rα and IL-2Rα (CD215 and CD25, respectively) (Olsen et al., 2007, Meghnem et al., 2017, Ma et al., 2021). IL-15 is made by different cells in the body. It plays an essential role in natural, adaptive immunity, inflammation, Natural Killer cell development, homeostasis and the stimulation of Cytotoxic T-cells. IL-15 is associated with autoimmune and inflammatory diseases, such as atherosclerosis, cardiovascular diseases (CVDs), the development of inflammation in malignancies, transplantation rejection, and infectious diseases (Panigrahi et al., 2020).

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Atherosclerosis is a chronic disease of the blood vessel wall characterized by the precipitation of plaques of fatty substances in and on inner artery walls, identified by an impactable interaction between the immune system and fats (Park and Park, 2015). The cells form plaque inflammation through a complex exchange among inflammatory factors in the circulation and artery wall. Hence, inflammation is a precursor of atherosclerotic plaque formation and a factor involved in atherosclerotic development (Ramji and Davies, 2015, Björkegren and Lusis, 2022). The activation of the inflammatory process in atherosclerosis and CVDs is mediated by the IL-15 cytokine, in all aspects. The overexpression of IL-15 in atherosclerosis and certain other CVDs promotes atherogenesis and may be an effective target in the treatment of CVDs (Guo et al., 2020). Other inflammatory factors, such as CRP and other cytokines, are the most prevalent intermediaries in the inflammatory process, have

been used in the medical diagnosis, and prognosis of the disease and have been usable targets for future therapies (Turillazzi et al., 2014).

Previous studies have demonstrated that IL-15 stimulates atherosclerotic plaque formation in mouse models (van Es et al., 2011). The values of IL-15 and soluble IL-15Rα are higher in the plasma of patients with CVDs than non-CVD individuals (Dozio et al., 2014).

As previously demonstrated, four mutations in IL-15 are related to atherosclerosis; however, the rs10833 variation may be significant in controlling IL-15 expression in atherosclerosis (Angeles-Martínez et al., 2017). Polymorphisms in the gene of IL-15 and IL-15 expression levels affect the development of coronary artery disease (Gokkusu et al., 2010).

Previous studies have demonstrated associations between an elevated hemoglobin A1c/ HDL–ratio and patients with atherosclerosis and carotid artery plaque with and without diabetes mellitus (Hu et al., 2021, Rossello et al., 2021). Alterations in serum CRP concentrations are associated with HbA1c values compared with fasting blood glucose values (Seo and Park, 2021). Others indicated that CRP and the ESR were good indicators of inflammation and atherosclerosis and were identified as vital risk factors and predictors of atherosclerosis (Xie et al., 2016, Koyama et al., 2015). Elevations in the serum levels of troponin T-hs and alkaline phosphatase have also been found to be related to an increased risk of developing atherosclerosis and several other CVDs (Ndrepepa, 2017, Li et al., 2021, Wang et al., 2021).

The present study aimed to determine whether IL-15 polymorphisms are related to risk factors for atherosclerosis in Iraqi patients. The analysis of IL-15 mutations may provide strategies with which to prevent the formation of atherosclerotic plaque and may also lead to the development of effective therapeutics.

2. Patients and Methods

2.1 Study Participants.

The present study was based on a case-control study design. Patients with atherosclerosis were enrolled from the Laboratory Department of the Surgical Specialty Hospital of Cardiac Center-Erbil (Erbil, Iraq). Patients who were admitted for CABG between April, 2021 and February, 2022 were incorporated in the current study. Venous blood samples were obtained from 87 cases with atherosclerosis (71 males and 16 females), median age 57 years and 30 healthy control individuals, their median age was 58.5 years. The patients with atherosclerosis were preferably diagnosed by the following clinical examinations: Echocardiography, cardiac assays, hemostasis, clinical chemistry screening tests and hematology analysis.

2.2 Blood Collection

Peripheral blood samples were collected by phlebotomy at the Hematology Laboratory, Surgical Specialty Hospital of Cardiac Center in Erbil-Iraq. subsequently, the venous blood samples were divided into two parts: One tube was used for serum biochemical analysis and the other for DNA extraction and sequencing. The latter was transferred to the Immunogen Center, in Erbil, Iraq.

2.3 Genotype Determination

The current study examined the genotypes of the IL-15 gene on exon 8 on the chromosomal location 4q31.21 (Imunogene Center, Erbil-Iraq). In accordance with the manufacturer's operating instructions, DNA was first extracted to obtain polymorphic mutation from blood cells of patients with atherosclerosis, using AddPrep (Genomic DNA Extraction kit Add Bio, Inc.) (Hamzei et al., 2020). DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). The PCR amplification was performed using the AddPrep kit according to the manufacturer's protocol with 35 amplification cycles. (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primers were used: IL-15 exon 8 forward, 5'-CTATGCTGGTAGGCTCCTG-3' and reverse,
5'-GTTCCATTAGAAGAGAGCTTGC -3'.

PCR was performed using the following steps: Denaturation for 5 min at 95 ℃; denaturation for 30 sec at 95˚C, annealing for 30 sec at 56˚C and elongation at 72˚C for 30 sec; a final elongation step was performed at 72˚C for 5 min. The DNA outcomes were isolated in one lane using 3% agarose gels and a reference 50 bp DNA ladder. Subsequently, they were dyed with Safe DNA Gel Stain dye (Add Bio, Inc.). The stained DNA bands were imaged using a UV light source with a UV Transilluminator (UST-20M-8K; Biostep GmbH) (Ghatak et al., 2013, Hamzei et al., 2020).

Following PCR, DNA extraction was performed to obtain the DNA samples for the sequencing process. The latter was performed using automatic gene analyzer 3130 Genetic analyzers (Applied Biosystems; Thermo Fisher Scientific, Inc.). Sanger sequencing was performed to obtain using the Mutation Surveyor software package 5 (5.0-UG001) (Soft Genetics, LLC) to detect known and unknown mutations by comparison with the GenBank database sequences reference genes (Chromosome 4 - NC_000004.12; - (https://www.ncbi.nlm.nih.gov/genbank)

2.4 Blood Analysis

Clinical chemistry analysis, which included blood sugar, lipid profile, electrolytes, kidney function and CRP levels) was performed using the Roche Cobas C311 analyzer. Thyroid function, serum ferritin, hemostasis [prothrombin time and international normalized ratio (INR)] and cardiac assays (CK-MB and troponin T-hs) were analyzed using the Roche Cobas e411 analyzer from the Laboratory Department of the Surgical Specialty Hospital of Cardiac Center-Erbil.

2.5 Statistical analysis

All statistical analyses were performed using GraphPad prism 8. The values were expressed as mean ± Standard Error and median. Normality was tested using the Anderson-Darling test, D'Agostino & Pearson test, Kolmogorov-Smirnov, and Shapiro-Wilk test. An independent samples (unpaired) t-test was used for blood parameters analysis

2.6 Ethics approval and consent to participate

The study was confirmed by the Human Ethics Committee of the College of Science, Salahaddin University-Erbil (Reference No: 7/54/590; date, 4/2/2021; Erbil-Iraq), and the study was carried out according to the principles of the Declaration of Helsinki. Permission was gotten from all patients and healthy subjects to obtain their blood before the CABG operation.

3. RESULTS AND DISCUSSION

The present study examined the effects of single nucleotide polymorphism (SNPs) of IL-15 on the biochemical and hematological parameters in patients with atherosclerosis.

3.1 GENOMIC VARIATION OF IL-15

In the current study, the genomic polymorphisms of IL-15 (exon 8) gene mutations in patients with atherosclerosis were analyzed using Mutation Survey software (Table 1).

A total of 4 mutations were obtained in exon 8 of the IL-15 gene at chromosome 4q31.21 in patients with atherosclerosis. The sequencing results of IL-15 identified one type of genomic polymorphism, which was substitution. Specifically, a total of four nucleotide substations were revealed in the IL-15 polymorphism genotypes with the following allele frequencies: (G>GA, G>GT, T>C and C>T). The variant percentage of the heterozygous mutation (97264G>GC) exhibited a frequency of 13.3% on the chromosome location 4:142654512 and the heterozygous variant (97270G>GT) a frequency of (26.7%) on chromosome location 4:142654518. The variant percentage of the homozygous mutation, 97299T>C (a frequency of 100%, location 4:142654547), and the homozygote variant 97553C>T (frequency of 53.3%, location 4:142654801) has been described in gene databases as dbSNP:10833 and as dbSNP:2291596, respectively.

However, the remaining two novel substitution variants of IL-15 were not recorded in the external databases, and no missense mutation record was reported in the present study. All IL15 mutations noted in patients with CVDs did not affect the amino acid sequence of the mentioned cytokine. These polymorphisms may affect serum IL15 production and influence atherosclerotic plaque formation and progression.

Previous studies have illustrated the presence of several variants in IL-15 that are significantly related to the development of atherosclerosis and inflammation, and SNP rs10833 may be involved in serum IL-15 production or may be associated with metabolic changes in atherosclerosis. It has been proposed that the association between the rs10833 variation and the IL-15 concentrations may be a mechanism through which this mutation affects disease development (Angeles-Martínez et al., 2017, Guo et al., 2020). The increased discharge of IL-15 in human blood vessel plaques promotes the development of microenvironments that support blood vessel endothelial dysfunction (Panigrahi et al., 2020).

Plasma concentrations of IL-15 and IL-15Rα appear to reflect body lipid distribution and epicardial adipose tissue. These phenomena occur due to the association between body lipid distribution, inflammation, genetic polymorphisms of the cytokine and CVDs (Dozio et al., 2014) (Jackson et al., 2018). The inflammatory reaction in arterial sclerosis is adjusted by both the natural and specific defense systems across the action of ILs (Ramji and

Davies, 2015). The elevation of serum troponin levels and the appearance of neurocardiogenic damage in a patient with stroke is considered to be mediated by epinephrine, norepinephrine and dopamine and endothelial dysfunction, which eventually leads to myocardial reappearing after initial damage (Sposato et al., 2020).

The risk of developing atherosclerosis is associated with elevated numbers of T-cells ($CD8^+$ cells) and the high expression of IL-15 in atherosclerotic plaque. An association has also been identified between signalling mechanisms, including tyrosine-protein kinase JAK1/STAT and Smad2/3 signalling, and IL-15 in the cardiovascular organs (Guo et al., 2020, Panigrahi et al., 2020, Björkegren and Lusis, 2022).

Table 1. Mutation observed in IL-15 gene in atherosclerosis patients analyzed with MutationSurveyor-5.0-UG001

	Chromosome Position	Mutation	Mutation genotype	Heterozygous / Homozygous	Variants	Variant Percentage	External Database
1	4:142654512	Substitution	G>GC	Heterozygous	97264G>GC	13.3%	Not Found
2	4:142654518	Substitution	G>GT	Heterozygous	97270G>GT	26.7%	Not Found
3	4:142654547	Substitution	T>C	Homozygous	97299T>C	100 %	dbSNP:10833
4	4:142654801	Substitution	C>T	Homozygous	97553C>T	53.3%	dbSNP:2291596

3.2 BLOOD ANALYSIS IN ATHEROSCLEROSIS

The most notable blood analysis results before CAGB in patients with atherosclerosis are presented in Table 2 and Figs. (1 and 2). The results revealed marked significant elevation in the median levels of fasting blood glucose (152.5; range, 104-219.8), mean HbA1c% levels (range, 7.300 ± 0.2277), alkaline phosphatase levels (P<0.05), ESR mm/h (P<0.05), median CRP levels 0.29 (range 0.14-0.6575) and troponin T-hs levels (P<0.01). Of note, the HDL level was significantly decreased (P<0.01) in the patients with atherosclerosis compared with the corresponding values noted in the healthy subjects. While, blood urea, creatinine, cholesterol, triglyceride, low-density lipoprotein (LDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, Na+, Ka+, Ca+2, serum ferritin, prothrombin time, INR, CK-MB, and thyroid function test not changed statistically in atherosclerosis disease. A previous study indicated that IL-15 expression, hs-CRP and HDL-cholesterol were related to the risk of circulatory system disease (Seo and Park, 2021).

Higher glycated hemoglobin concentrations have also been found to be associated with an elevated risk of developing atherosclerosis in individuals who are at low risk of CVDs. The HbA1c and fasting blood glucose levels can be used as diagnostic biomarkers of CVDs. Increased HbA1c and decreased HDL-cholesterol levels are also significantly associated with the development of coronary artery disease in cases with or without diabetes (Rossello et al., 2021, Hu et al., 2021). The induced production of free radicals from glycation end products may also be a pro-inflammatory effect of hyperglycemia or an increased glucose level affects the nuclear factor-kB mechanism through the expression of pro-atherosclerotic and proinflammatory genes in blood vessel cells (Orasanu and Plutzky, 2009, Chehaibi et al., 2016).

The elevation of alkaline phosphatase levels is associated with blood vessel calcification and the direct contribution of the alkaline phosphatase to the pathogenesis of atherosclerosis (Ndrepepa, 2017, Hansson, 2005). Alkaline phosphatase is associated with CRP in CVDs. Therefore, inflammation plays a vital role in the initial phases of atherosclerotic plaque formation, from fixed to unfixed plaque and ensuing clinical syndrome (Ndrepepa, 2017, Hansson, 2005). Elevated ALP expression leads to blood vessel calcification and

stimulates the atherosclerosis process via hydrolyzing inhibitor of vascular calcification which is inorganic pyrophosphate (Kunutsor et al., 2015).

Previous studies have confirmed that a high concentration of the heart troponin T-hs is a hazard factor for CVDs and atrial fibrillation in atherosclerosis (Wang et al., 2021, Li et al., 2021). The troponin T-hs concentration is significantly associated with CRP and ESR. CRP is an important biomarker in the advancement of CVDs. Blood artery or heart injury results in the release of tissue proteins and may be recognized by the body's immune system, leading to the production of autoimmune antibodies against these proteins. Immune dysfunction and inflammatory conditions appear to be critical factors in atherosclerosis (Abdelaziz et al., 2021, Chaulin, 2021a, Chaulin, 2021b, Kaya et al., 2010).

Table 2. Blood analysis in individual with and without Atherosclerosis

Parameters	Patients with Atherosclerosis	Control without Atherosclerosis	P-Value
Glucose (mg/dl)	152.5 (104-219.8)	106.3 (98.5-117.8)	0.01
HbAc1 (%)	7.300 ± 0.2277	6.156± 0.1375	0.05
Urea (mg/dl)	45.00 (42.25-52.75)	36.00 (35.00+47.00)	NS
Creatinine (mg/dl)	0.8600 (0.7400- 1.068)	0.8200 (0.7850- 0.91)	NS
Cholesterol (mg/dl)	132.0 (109.5 - 145.8)	142.5±13.1	NS
Triglyceride (mg/dl)	131.0 (106.0-139.3)	127.9 ± 7.477	NS
HDL-Cholesterol (mg/dl)	37.50 (31.75-43.00)	45.20±1.884	0.01
LDL-Cholesterol (mg/dl)	76.50 (53.75-99.75)	97.50 (51.50-105.0)	NS
AST (GOT) U/L	18.50 (14.50-24.25)	21.20 ± 1.652	NS
ALT (GPT) U/L	14.50 (10.75-26.00)	20.60 ± 1.694	NS
Alkaline Phosphatase (U/L)	89 (72-106.3)	72.00 ±4.450	0.05
T. Bilirubin (mg/dl)	0.7150 (0.315+0.855)	0.380 (0.265-0.80)	NS
Na (mmol/L)	138.0 (136.8-139.5	139.0 (137.5-140.3)	NS
Ka (mmol/L)	4.500 (4.175-4.825)	4.560+ 0.1477	NS
Ca (mmol/L)	1.285 (1.215-1.33)	1.28 (1.228-1.333)	NS
CRP (mg/dl)	0.29.5 (0.14-0.6575)	0.14 (0.13-0.21)	0.05
ESR (mm/1hr)	16 (7-34)	8 (5-14.25)	0.05
S. Ferritin (ng/ml)	123.3 (54.69-205.8)	115 (55.74-200.7)	NS
Prothrombin Time (PT) (Sec.)	14.80 (13.45-15.03)	13.29 ± 0.2433	NS
International Normalized Ratio (INR)	1.1 (0.9925-1.123)	1.03 (0.993-1.133)	NS
CK-MB (ng/ml)	2.245 (1.495-6.345)	2.666±0.4969	NS
Troponin T-hs (ng/ml)	0.0165 (0.0077-0.03475)	0.0087± 0.00202	0.01
T3 (nmol/L)	1.57(1.465-1.83)	1.71 (1.555-1.825)	NS
T4 (nmol/L)	116.7±8.796	104.3 (99.7-118.3)	NS
TSH (uIU/ml)	2.77 (1.223-4.218)	2.25 (0.9668-4.39)	NS

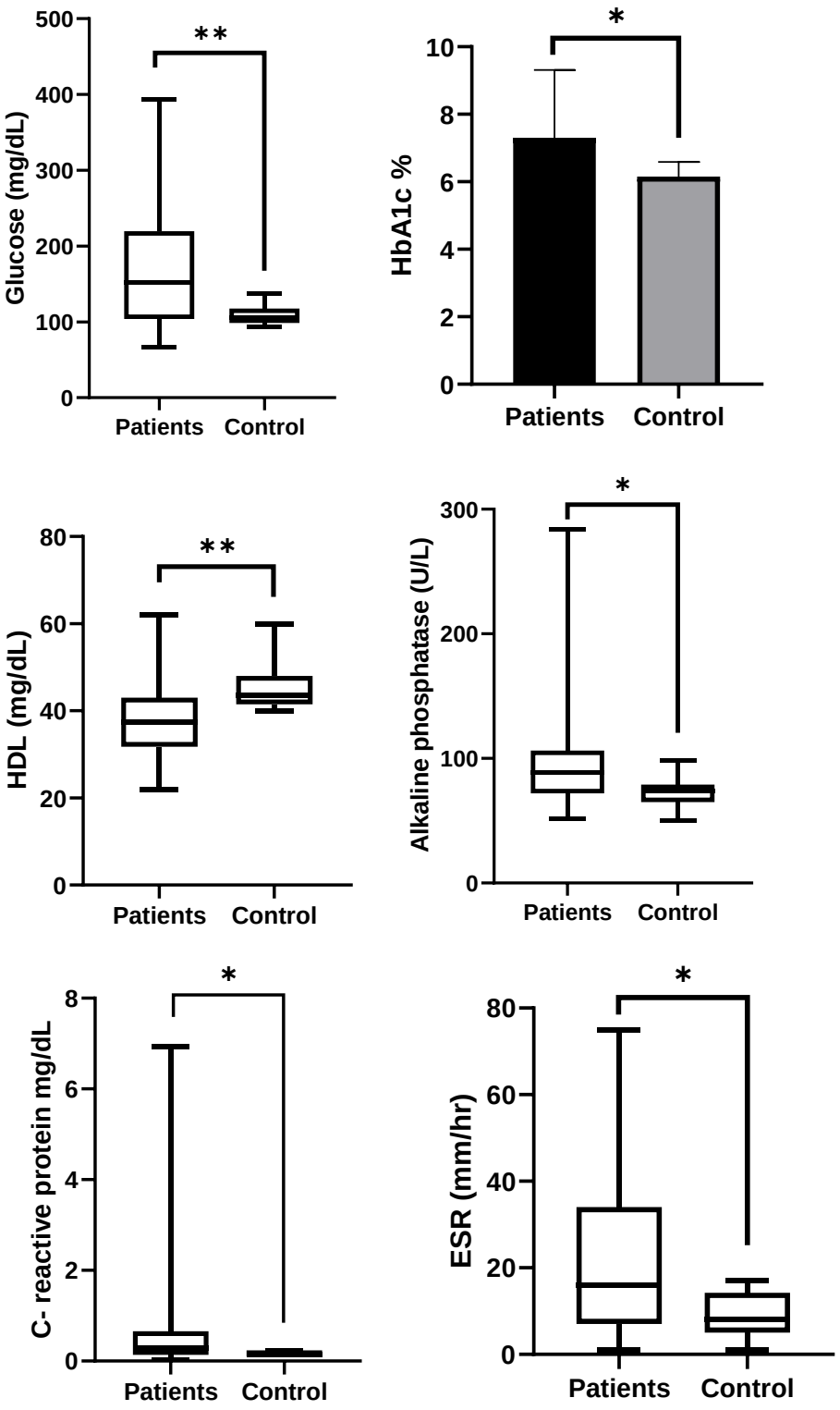

Figure (1). Shows some biochemical and hematological criteria in atherosclerosis patients

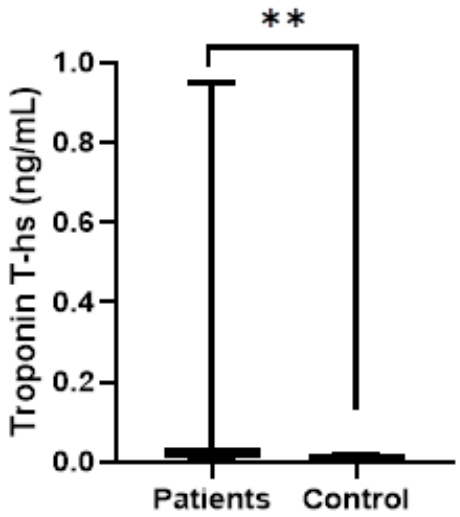

Figure (2). Shows cardiac assays criteria in atherosclerosis diseases

CONCLUSION

In conclusion, the present study identified associations between several novel polymorphisms in IL-15 and risk factors for atherosclerosis disease. The data revealed a relationship between rs2291596, rs10833 and two other novel mutations with artery vessel atherosclerosis. In addition, a significant association was found between HbA1c, fasting serum glucose, HDL, ESR, CRP, troponin T-hs and alkaline phosphatase values with the disease.

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CONFLICTS OF INTEREST: None

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