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Unraveling the Mystery: How High Density Lipoprotein 'Good' Cholesterol Goes 'Bad'?

Gizemi Çözmek: Yüksek Dansiteli Lipoprotein 'İyi' Kolesterol Nasıl 'Kötü' Oluyor?

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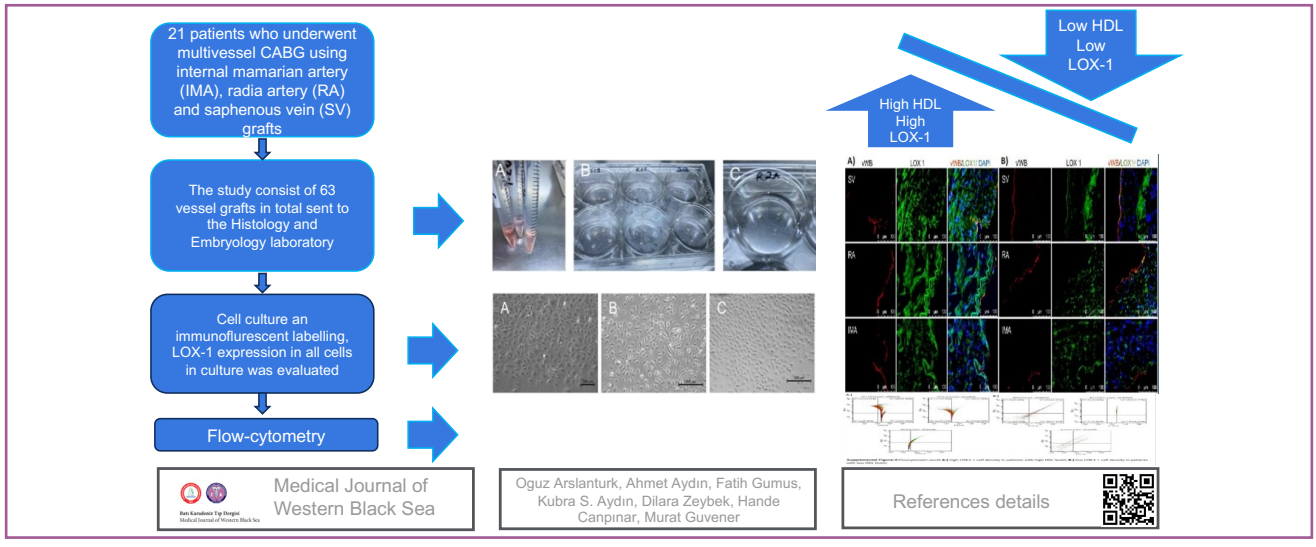
GRAPHICAL ABSTRACT



ABSTRACT

Aim: Recent research has underscored the critical importance of Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in the development of atherosclerosis, especially in relation to its interaction with high-density lipoprotein (HDL). This study focused on exploring the role of dysfunctional HDL in binding to LOX-1 in patients with Coronary Artery Disease (CAD) and its potential impact on the progression of atherosclerosis.

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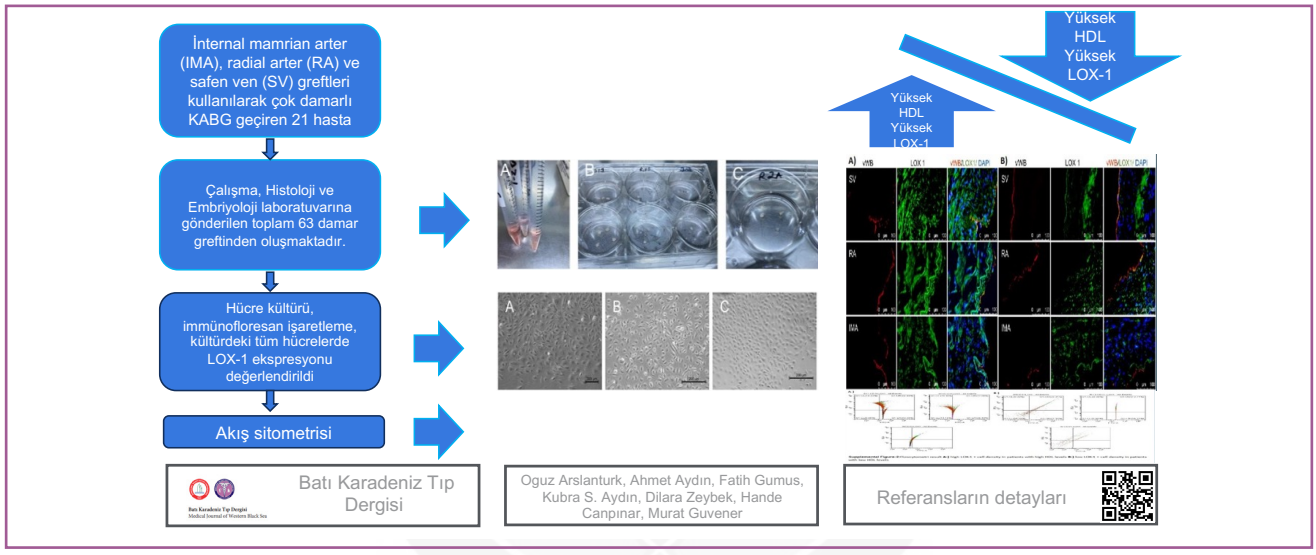
Material and Methods: This prospective, single-center study, conducted between November 2019 and April 2020, included 21 patients who underwent multivessel coronary artery bypass grafting (CABG). Endothelial cells, isolated from the collected vessel specimens, were cultured in EGMTM-2 medium. LOX-1 expression was measured using flow cytometry and immunofluorescence labeling. The intracellular localization of LOX-1 was analyzed alongside Von Willebrand Factor (vWB) and LDL receptor to elucidate LOX-1's role in vascular pathology.

Results: The study included 21 patients, of whom 17 (80.9%) were male and 4 (19.04%) were female. A significant positive correlation was found between HDL levels and LOX-1 expression in all graft types (saphenous vein (SV), r=0.60; radial artery (RA), r=0.48; internal mammary artery (IMA), r=0.53). HDL accounted for approximately 28% of the variation in LOX-1 expression for IMA (F=7.03; p<0.05), 36% for SV (F=10.10; p<0.05), and 24% for RA (F=5.64; p<0.05).

Conclusion: The findings suggest that elevated LOX-1 receptor expression may impair high-density lipoprotein (HDL) functionality by promoting its binding to LOX-1. Monitoring both HDL and LOX-1 levels could enhance the prediction of treatment responses and guide the selection of suitable therapeutic interventions.

Keywords: Atherosclerosis, coronary artery bypass grafts, cardiovascular disease, lipoprotein

GRAFİKSEL ÖZET



ÖZ

Amaç: Son yıllarda, yapılan araştırmalar, aterosklerozun ilerlemesinde Lektin-benzeri oksitlenmiş düşük yoğunluklu lipoprotein reseptör-1'in (LOX-1) önemini, özellikle yüksek yoğunluklu lipoprotein ile etkileşimi açısından vurgulamıştır. Bu çalışmada, koroner arter hastalığı (KAH) bulunan hastalarda disfonksiyonel yüksek yoğunluklu lipoproteinin (HDL) LOX-1'e bağlanma rolü ve bunun aterosklerozun ilerlemesine olan etkileri araştırılmıştır.

Gereç ve Yöntemler: Bu prospektif tek merkezli çalışmaya Kasım 2019 ile Nisan 2020 tarihleri arasında çok damar koroner arter baypas grefti (KABG) uygulanan 21 hasta dahil edildi. Endotel hücreleri damar örneklerinden izole edildi ve EGMTM-2 ortamı kullanılarak kültürlendi. LOX-1 ekspresyonu, akış sitometrisi ve immünofloresan etiketleme kullanılarak değerlendirildi. Endotel hücreleri, Von Willebrand Faktörü (vWB) ve düşük dansiteli lipoprotein reseptörü ile birlikte LOX-1'in hücre içi lokalizasyonu açısından değerlendirilerek, LOX-1'in vasküler patolojideki mekanik etkilerine ilişkin bilgiler sağlandı.

Bulgular: Çalışmamız, 17'si (%80.9) erkek ve 4'ü (%19.04) kadın olmak üzere 21 hastadan oluşmaktadır. Tüm greft türlerinden alınan örneklerde yüksek dansiteli lipoprotein ve LOX-1+ ifadesi arasında anlamlı bir pozitif korelasyon gözlemledik (Safen Ven, r=0.60; Radial arter, r=0.48; İnternal mamarian arter, r=0.53). IMA için LOX-1+ ifadesindeki varyasyonun yaklaşık %28'i (F=7.03; p<0.05), SV için %36'sı (F=10.10; p<0.05) ve RA için %24'ü (F=5.64; p<0.05) yüksek dansiteli lipoproteine atfedildi.

Sonuç: Bulgularımız, yüksek LOX-1 reseptör ifadesi olan durumlarda yüksek dansiteli lipoproteinin LOX-1'e bağlanarak işlevselliğini kaybettiğini öne sürmektedir. yüksek dansiteli lipoprotein ve LOX-1 seviyelerinin izlenmesi, hastaların tedaviye yanıt verme tahminini artırabilir ve uygun müdahaleleri belirlemeye yardımcı olabilir.

Anahtar Sözcükler: Ateroskleroz, koroner arter bypas greftleri, kardiyovasküler hastalık, lipoprotein

INTRODUCTION

The World Health Organization (WHO) identifies Cardiovascular Disease (CVD) as the leading cause of death on a global scale (1). CAD is a clinical syndrome that impairs coronary blood flow in the myocardium as a result of atherosclerotic lesions. Atherosclerosis can be defined as narrowing of the artery due to plaque formation in lipid accumulation within arterial walls (2).

While current medical therapy effectively addresses CAD, a considerable number of patients necessitate revascularization. CABG emerges as a potent approach to alleviate or eradicate angina pectoris symptoms. The long-term success of bypass surgery is influenced by the type of graft used (saphenous vein, radial artery, or internal mammary artery) and the advancement of atherosclerosis in the native coronary vessels. There are many factors related to progression of atherosclerosis and studies have shown that low HDL levels is one of the most significant risk factor (3, 4). Recent research indicates that higher HDL levels might be linked to an increased risk of Coronary Artery Disease (CAD). This suggests that HDL's protective role against cardiovascular events is not solely dependent on its concentration. (5-7).

The American Heart Association (AHA) has advocated for the enhancement of overall cardiovascular health through the promotion of seven components of ideal cardiovascular health. These components include health behaviors such as abstaining from smoking, engaging in regular exercise, and maintaining a healthy diet, as well as health factors like achieving an ideal body mass index, optimal cholesterol levels, blood pressure control, and blood glucose management (8). LOX-1 (Lectin-like oxidized low-density lipoprotein receptor-1), a transmembrane protein receptor, plays a crucial role in the pathogenesis of cardiovascular diseases, including atherosclerosis, endothelial dysfunction, and thrombosis. It contributes to the formation of foam cells by macrophages through the uptake of cholesterol esters in oxidized lipoproteins, thereby promoting the development of atherosclerotic plaques (9). Several studies emphasize the crucial role of LOX-1 in atherosclerosis and related diseases. LOX-1 also serves as a receptor for modified HDL, and its overexpression results in HDL binding, leading to dysfunction and promoting proatherogenic effects (10).

In this study, we aimed to demonstrate the role of non-functional HDL binding to LOX-1 in the progression of atherosclerosis among patients with CAD.

MATERIAL and METHODS

Study Population

The study protocol was approved by the Hacettepe University Ethics Evaluation Committee (GO 18/1134) on May 7, 2019. Written informed consent was obtained from all participants and their relatives. This prospective, single-center study included 21 patients who underwent multivessel CABG using internal mammary artery (IMA), radial artery (RA), and saphenous vein (SV) grafts between November 2019 and April 2020. Remaining vessel fragments were used for specimen analysis. Endothelial cells were marked with CD31, and LOX-1 expression was evaluated in all cultured cells. Patients who declined, did not undergo CABG with these grafts, or were under 18 were excluded.

Surgical Procedures and Materials

All patients were operated under general anesthesia, placed in a supine position and midline split sternotomy was performed. The LIMA, radial artery and greater saphenous vein were harvested in standard fashion. Each specimen from the related graft was taken from the most unused distal portion. The collected materials were isolated from adipose tissue and foreign matter, then transported to the Histology and Embryology laboratory in a medium comprising Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin.

Cell Culture – Endothelial Cell Isolation

The specimens in the transport medium containing DMEM + 1% penicillin-streptomycin were transferred to a petri dish and rinsed with Dulbecco's phosphate-buffered saline (DPBS) to eliminate heparin. A portion of the incoming tissue samples was cryopreserved and stored in liquid nitrogen, while the remaining fragments were cut into pieces averaging 2x2mm under sterile conditions in a laminar flow cabinet. These pieces were then placed onto 6-well and 12-well plates pre-coated with Geltrex (A15696-01, Thermo Fisher Scientific, Rockford, IL). EGMTM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKitTM (CC-3202, Lonza, Switzerland), specifically designed for endothelial cells, was added to the fragments, allowing 3-4 hours for adherence. Subsequently, 1500 μl of EGMTM -2 medium was dispensed per well into 6-well plates, and 1000 μl per well into 12-well plates. The primary culture was then incubated at 37°C with 5% CO2, with medium changes occurring at 2-3 day intervals. After 1 week, cells were observed under a phase contrast microscope, and the tissue pieces were removed from the wells. Culture continued until the endothelial cells reached 80% confluence. Cells from the 12-well plates were employed for immunofluorescent labeling, while those from the 6-well plates were used for flow-cytometric evaluations. Trypsin-EDTA was applied to cells in 6-well plates once they reached 80% confluence, followed by centrifugation at 1200 rpm for 5 minutes. The supernatant was then discarded, and the remaining pellet was resuspended in 1 mL PBS for flow cytometry analysis.

Flow Cytometry

LOX-1+ expression in cultured endothelial cells was assessed using flow cytometry. Cells were aliquoted at $1x10^6$ cells per ml, followed by the addition of 5 μl LOX-1 PE antibody (Biolegend clone 15C4) and a 30-minute incubation at +4°C. Post-incubation, cells were washed with PBS, centrifuged, and resuspended in 0.5 ml PBS. Flow cytometry (Beckman Coulter, Cytoflex USA) was used to count 10,000 cells. Cells were gated by size (Forward Scatter) and granularity (Side Scatter), and fluorescence intensity was analyzed to determine the percentage of LOX-1+ cells, accounting for non-specific binding with isotype controls.

Immunofluorescent Labelling

Endothelial cells cultured to 80% confluence were labeled for Von Willebrand Factor (vWB), LDL receptor, and LOX-1 using double immunofluorescence. Cells were fixed, permeabilized, and blocked before being incubated with primary antibodies and secondary antibodies (Alexa Fluor® 594 and Alexa Fluor® 488). Nuclei were stained with DAPI, and imaging was performed using a fluorescence microscope.

For tissue samples, 7μm sections were cut, acetone-fixed, and labeled with vWB/LOX-1. After blocking, primary and secondary antibodies were applied, followed by DAPI staining. Sections were imaged with a fluorescence microscope.

Statistical Analysis

Data were reported as percentages, numbers, or mean ± SD. Normality was checked with the Shapiro-Wilk test, and ANOVA was used for normally distributed variables. Pearson's correlation was categorized from weak to very strong, and beta coefficients were derived via univariate linear regression. Analysis was performed using SPSS version 23.0, with significance at $p < 0.05$.

Table 1: Demographic and clinical characteristics of all patients.

Characteristics	Findings (n=21)
Age (Years)*	65.71±9.63 (45-83)
Gender, n (%)	
Male	17 (81)
Female	4 (19)
Total Cholesterol (mg/dL) *	157.63±48.95 (95-249)
HDL-c (mg/dL) *	36.38±11.20 (21-69)
LDL-c(mg/dL) *	112.74±36.49 (69-165)
Total Cholesterol /HDL- (ratio) *	4.38±1.36 (2.04-7.20)
Diyabetes, n (%)	10 (47.6)
Hypertension, n (%)	15 (71.4)
Hyperlipidemia, n (%)	13 (61.9)

* Data are shown as means ±Standart Deviation (minimum-maximum).

RESULTS

The demographic and clinical characteristics of the cases were demonstrated in Table 1 with details. The study consists of 21 patients, 17 (80.9%) males and 4 (19.04%) females. The mean age is 65±9. The study consists of 63 vessel grafts in total.

A few adherent cells were observed around the tissue pieces on the 5th day after explant culture made from SV, RA and IMA tissue samples. In the following days, these cells were observed to be endothelial cells that proliferated and adhered as a more rounded monolayer.

Although the appearance of endothelial and fibroblast cells is different under phase contrast microscopy, specific markers should be used to differentiate cells precisely. For this reason, CD31 and vWB factor, which are specific for endothelial cells, were used in flow cytometry and immunofluorescence labeling.

The positive immunofluorescent labeling of vWB/LDL and vWB/LOX1 was observed in cells grown as monolayer after explant culture from SV, RA and IMA. Granular labeling of vWB was observed in the cytoplasm of endothelial cells. vWB and LDL were positive together in culture cells obtained from the SV, RA and IMA. LOX1 immunoreactivity was observed punctually in the cytoplasm of endothelial cells.

In the sections of SV, RA and IMA samples, vWB/LOX-1 positive endothelial cells were observed on the surface facing the lumen. LOX1 immunflourescence was also observed on fibroblasts and smooth muscles. LOX1 immunreactivity was strong in the samples with high HDL level and LOX1 immunreactivity was weak to moderate in the samples of low HDL level (Figure 1).

When comparing participants with Saphenous/radial and IMA grafts, no significant differences were observed in CD31+LOX-1+1, LOX-1+, and CD31+ & Low-density lipoprotein receptor (LDLR)+ measurements (Table 2). However, correlation analysis revealed a strong positive correlation between HDL and LOX-1+ in specimens from all types of grafts (SV, r=0.60; RA, r=0.48; IMA, r=0.53). Additionally, a strong positive correlation was found between HDL and CD31+ & LDLR+ in IMA grafts (r=0.683), and a positive correlation between LDL and LOX-1+ in SV grafts (r=0.479) (Table 3).

Furthermore, a strong positive correlation was observed between HDL levels and LOX-1+ expression in saphenous vein (SV) specimens (r=0.600). Univariate linear regression analysis revealed that approximately 36% of the variation in LOX-1+ expression in SV specimens could be attributed to HDL levels ($F=10.10$; $p<0.05$). Specifically, a one-unit increase in HDL was associated with a 0.73 increase in LOX-

1+ expression in SV graft specimens. The corresponding model to estimate LOX-1+ based on HDL in SV specimens was YLOX-1+ (SV) = 2.35 + 0.73*X HDL. Similar calculations were conducted for other graft types.

Table 2: Comparison of the receptor percentage of cells in the saphenous vein/ radial artery/ internal mammarian artery.

Comparison of the receptor percentage of cells		Findings (n=21)	p
CD31+ &LOX-1+*	Saphenous vein	42.76±15.65 (12-69)	0.550
	Radial artery	42.10±18.04 (20-86)	
	IMA	41.10±12.79 (21-68)	
LOX-1+*	Saphenous vein	28.71±13.32 (8-60)	0.123
	Radial artery	35.52±18.68 (14-90)	
	IMA	31.81±15.33 (12-66)	
CD31+ & LDLR+*	Saphenous vein	32.90±12.38 (15-63)	0.952
	Radial artery	31.05±12.42 (12-68)	
	IMA	30.90±11.67 (9-56)	

* Data are shown as means ±Standart Deviation (minimum-maximum). P value was obtained from ANOVA test. **IMA:** Internal mammarian artery

A moderate positive correlation was found between HDL levels and LOX-1+ expression in radial artery (RA) specimens (r=0.488). Univariate linear regression analysis showed that about 24% of the variance in LOX-1+ in RA specimens could be accounted for by HDL levels (F=5.64; p<0.05). Specifically, each unit increase in HDL corresponded to a 0.80 increase in LOX-1+ expression for RA graft specimens. Therefore, the predictive model for LOX-1+ based on HDL in RA specimens is YLOX-1+ (RA) = 5.30 + 0.80*X HDL.

Similarly, in internal mammary artery (IMA) specimens, a moderate positive correlation was noted between HDL and LOX-1+ (r=0.488). Univariate linear regression analysis demonstrated that approximately 28% of the variation in LOX-1+ in IMA specimens was explained by HDL (F=7.03; p<0.05). A one-unit increase in HDL led to a 0.71 increase in LOX-1+ for IMA graft specimens, leading to the formula YLOX-1+ (IMA) = 4.99 + 0.71*X HDL (Table 3).

DISCUSSION

The long-term effectiveness of coronary artery bypass grafting (CABG) surgery is influenced by the choice of graft material and the advancement of atherosclerotic disease in the

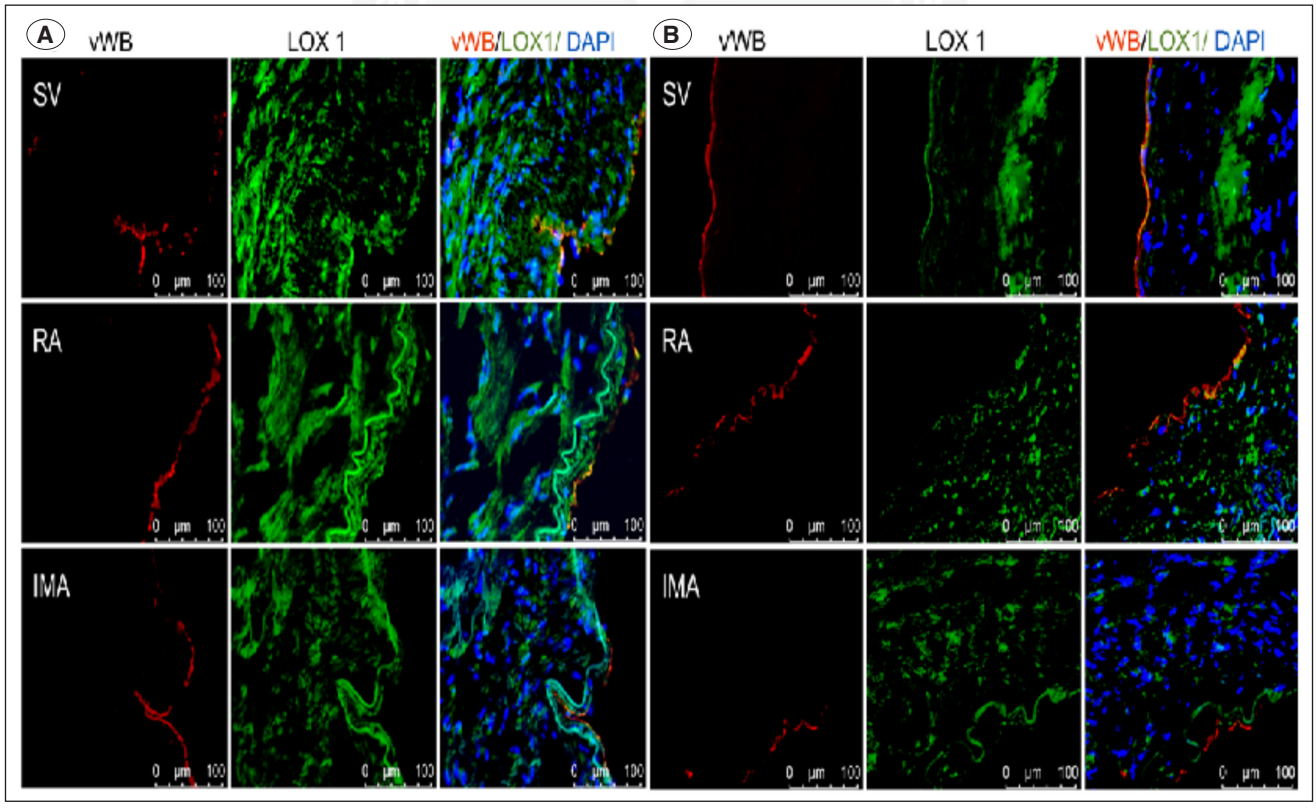


Figure 1: LOX-1 expression in the samples with HDL level. **A)** LOX-1 immunreactivity in the sections of saphenous vein (SV), radial artery (RA) and internal mammarian artery (IMA) of the samples with high HDL level (HDL>40). **B)** Representative micrographs showing weak to moderate LOX-1 immunreactivity in the section of SV, RA and IMA of the samples with low HDL level (HDL<40). vWB in red, LDL receptor in green, LOX-1 in green, DAPI nuclear stain in blue. vWB and LOX-1 positive endothelial cells.

Table 3: Correlation analysis of clinical parameters of patients with LOX-1+ in saphenous vein, radial artery and internal mammarian artery.

		CD31+&LOX-1+			LOX-1+			CD31+ & LDLR+		
		SV	Radial	IMA	SV	Radial	IMA	SV	Radial	IMA
Age	r	-0.404	-0.111	-0.112	-0.220	-0.112	-0.041	-0.097	-0.086	-0.123
	p	0.070	0.633	0.628	0.339	0.629	0.861	0.675	0.710	0.594
Total cholesterol	r	0.115	-0.119	0.332	0.439	0.018	0.281	0.192	-0.023	0.355
	p	0.528	0.626	0.165	0.060	0.943	0.244	0.431	0.925	0.136
HDLc	r	0.065	0.155	0.414	0.600**	0.488*	0.530*	0.372	0.299	0.683**
	p	0.785	0.513	0.070	0.005	0.029	0.016	0.106	0.200	0.001
LDLc	r	0.139	-0.120	0.258	0.479*	-0.030	0.343	0.141	-0.025	0.366
	p	0.560	0.615	0.272	0.032	0.900	0.139	0.554	0.915	0.113
Total cholesterol / HDLc	r	0.082	0.043	-0.051	-0.088	-0.161	-0.065	-0.134	0.003	-0.203
	p	0.746	0.866	0.841	0.727	0.524	0.797	0.595	0.989	0.419

** p<0.001 * p< 0.05 r:Pearson corelation coefficent, sd, standard deviation (n=21), **SV:** Saphenous vein, **IMA:** Internal mammarian artery

patient's native coronary arteries. Key risk factors for the development of atherosclerosis in grafts include smoking, hypertension, diabetes, high cholesterol levels, and obesity (11). Regarding long-term patency rates among different grafts, studies have conclusively shown that using an internal thoracic artery can enhance life expectancy compared to other vascular grafts, with long-term patency rates ranging from 10 to 20 years (12).

Despite numerous factors influencing the patency of implanted grafts in patients undergoing CABG, some reports suggest that LOX-1 plays a vital role in atherosclerotic and related diseases (10). LOX-1 serves as a receptor for modified HDL, and overexpression of LOX-1 binding to HDL renders it dysfunctional and imparts proatherogenic properties. However, in our current study, we couldn't establish the superiority of IMA in terms of LOX-1 receptor expression rates; no significant difference was found between the grafts (p>0.05), despite IMA demonstrating the highest patency and lowest atherogenic properties. LOX-1 expression is highly regulated and significantly upregulated by various stimuli. Cellular LOX-1 expression is also increased in patients with risk factors for atherosclerosis, including hypertension, diabetes, hypercholesterolemia, and smoking (10). Therefore, assessing the response after transplantation is crucial. We believe that evaluating the percentage of LOX and LDLR with homogeneous large groups through randomized controlled studies in the future would provide more insights. Additionally, it's essential to separate all cell types in the analyzed cell cultures and isolate each type of cell separately. In this study, we isolated only endothelial cells. Particularly in arterial grafts, arteriosclerosis progresses to some degree, and proliferating smooth muscle cells express LOX-1 (13). However, analyzing LOX-1 expression in all cultured cells would likely add further depth to the study.

LOX-1 actively contributes to all stages of atherogenesis and has been found to be expressed not only in endothelial cells but also in macrophages, vascular smooth muscle cells, and platelets. With the binding of ox-LDL, formed as a result of oxidative modification of LDL, to LOX-1, nitric oxide (NO) catabolism increases, NO secretion decreases due to weakened endothelial NO synthase activity, leading to endothelial dysfunction and atherosclerosis (13). Basal expression of LOX-1 in endothelial cells is limited in vitro, but it can be rapidly induced by proinflammatory, prooxidative, and mechanical stimuli (14). Basal expression of LOX-1 is also low in vivo, but its expression increases with various pathological conditions, including hypertension, diabetes mellitus, hyperlipidemia, chronic renal failure (13). Studies have shown that mice with both LDLR and LOX-1 deletions exhibit more atherosclerosis than those with isolated LDLR deletions (15). Furthermore, mice with only LOX-1, only LDLR, and LDLR/LOX-1 double deletions have been examined, with mice exhibiting isolated LOX-1 deletion showing less collagen deposition in atherosclerotic plaque (16). Research has also demonstrated a strong positive correlation between ox-LDL levels and the severity of acute coronary syndromes (17).

Recent studies have highlighted the complex relationship between soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) and CAD (18, 19). Given the inherent presence of CAD within our study cohort, characterized by elevated sLOX-1 levels, we opted to explore the correlation between HDL levels and LOX-1 receptors derived from vascular endothelial cell cultures.

While the mechanism of atherosclerosis and the crucial effect of LDL and LOX-1 have been clearly elucidated in studies, the effect of other types of lipoproteins, especially HDL, and their relationship with LOX-1, remains undefined in the

literature. Although there is a known inverse correlation between HDL cholesterol concentration and CAD, recent epidemiological studies suggest that very high concentrations of HDL cholesterol may increase the risk of CAD (5, 7, 20). Moreover, a randomized controlled study demonstrated that genetically high plasma HDL concentrations do not reduce the risk of myocardial infarction (21). Besler et al. reported the inability of HDL taken from patients with CAD to stimulate endothelial NO production, indicating the loss of antiatherogenic property of HDL (22). This could be explained by the LOX-1 binding of modified HDL (22). Madsen et al. also found that in individuals with exceptionally high HDL cholesterol levels, the conformation and functional properties of HDL particles may be altered, potentially impairing their normal function and leading to adverse effects. In our study, we observed a significant positive correlation between HDL levels and the percentage of LOX-1+ receptors in all three graft cultures, indicating the presence of dysfunctional HDL in these patients. Similar observations have been reported in recent large-scale clinical studies, where elevated HDL levels did not reduce the risk of cardiovascular diseases (7, 23-26). These findings imply that, in patients with CAD, higher HDL levels may bind to LOX-1, compromising its presumed antiatherogenic function. As far as we know, this study is the first to individually demonstrate the correlation between LOX-1 receptors and HDL in grafts utilized for CABG.

The primary limitation of this study is the absence of comparative data, given that all participants were diagnosed with CAD. Another limitation is that each cell type was not isolated in cell cultures, and the number of patients was insufficient to evaluate their sub-parameters.

Our study identified a positive correlation between LOX-1 and HDL levels. This finding indicates that in instances of elevated LOX-1 receptor expression, HDL, which is usually considered protective against atherosclerosis, may bind to LOX-1 and subsequently lose its functional properties. Therefore, it may not be adequate to assess the risk of cardiovascular disease solely based on HDL levels. Measuring HDL dysfunction could be more crucial in evaluating CVD risk. Additionally, it may be possible to demonstrate the long-term outcomes of graft patency through the LOX-1 receptor.

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Author Contributions

Conceptualization: **All authors,** Methodology: **Oguz Arslanturk, Kubra Aydın, Dilara Zeybek, Hande Canpinar, Murat Guvener,** Investigation: **Oguz Arslanturk, Murat Guvener,** Data Curation: **Oguz Arslanturk, Kubra Aydın, Dilara Zeybek, Hande Canpinar, Murat Guvener,** Original Draft Preparation: **All authors,** Writing, Review & Editing: **Oguz Arslanturk, Fatih Gumus, Murat Guvener,** Visualization: **All authors,** Supervision: **Murat Guvener.**

Conflicts of Interest

The authors have no financial disclosures that would be a potential conflict of interest with the current manuscript. Oral Presentation: Arslanturk O. "Protective Effect of HDL Against Coronary Artery Disease: LOX-1 Binding HDL is a Devil" 18th Congress of Innovations in Cardiology and Cardiovascular Surgery, 1-4 December 2022, Antalya, Türkiye.

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Ethical Approval

The study protocol received approval from the Hacettepe University Ethics Evaluation Committee (Approval No. GO 18/1134) on May 7, 2019. Written informed consent was obtained from all patients and, where applicable, from their relatives.

Review Process

Extremely and externally peer-reviewed.

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