Lectin-like oxidised LDL receptor-1 as a marker of endothelial dysfunction in Behçet's disease

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ABSTRACT

Objective. The aim of this study was to examine the distribution of lectin-like oxidised LDL receptor-1 (LOX-1) levels in patients with active BD, possible association of LOX-1 with the oxidised LDL (oxLDL), endothelial nitric oxide synthase (eNOS), nitric oxide (NO), endothelin-1 (ET-1) levels, and to characterise the differences between patients with active BD and those with systemic lupus erythematosus(SLE) in terms of these parameters compared with healthy controls.

Methods. A total of 30 patients with active BD, 22 patients with SLE as patients controls, and 30 healthy subjects were enrolled in this study.

Results. Significantly lower eNOS ve NO levels were observed in patients with BD and SLE compared with healthy controls. oxLDL, LOX-1 ve ET-1 levels were significantly increased in active periods of patients with BD and SLE compared with healthy control. There was no significant difference in oxLDL levels between subjects with BD and SLE. LOX-1 levels were significantly higher in active periods of patients with BD than in SLE, ET-1 levels were significantly lower.

Conclusion. Endothelial dysfunction parameters are elevated in patients with BD having active disease. The necessary measures should be considered in terms of risk of atherosclerosis in BD, especially for the early identification of endothelial damage by looking at LOX-1 levels.

Introduction

Behçet's disease (BD) is a multisystem disorder characterised by opthalmic and dermatologic findings, mucocutenous lesions, neurologic findings, arthritis and cardiovascular system involvement. BD is a chronic recurrent inflammatory disease, and its predominant histopathology is vasculitis in which perivascular tissue is infiltrated by predominantly lymphocytes and monocytes, plasma cells, and neutrophils as well. Cronic inflammation is a important risk factor in the pathogenesis of atherosclerosis and accelerated atherogenesis has previously been shown in inflammatory rheumatic diseases such as systemic lupus erythematosus (SLE) (1-7).

Endothelial dysfunction is an indicator for vascular involvement in any disease affecting the vascular structure, which can be linked with the vascular involvement in BD (4). Endothelial dysfunction and oxidised LDL (oxLDL) is believed to play a key role in the initiation of the atherosclerotic process. Lectin-like oxidised low-density lipoprotein receptor (LOX)-1 is a type II membrane protein that belongs to the C-type lectin family of molecules, which can act as a cell-surface endocytosis receptor for atherogenic oxLDL. LOX-1 has been identified as a major receptor for oxLDL in endothelial cells, monocytes, platelets, cardiomyocytes, and vascular smooth muscle cells. Measurement of soluble LOX-1 (sLOX-1) *in vivo* may provide a novel diagnostic tool for the evaluation and prediction of atherosclerosis and vascular disease (8). sLOX-1 is identified endothelial receptor for oxLDL that plays a pivotal role in oxLDL-induced endothelial dysfunction (9).

Nitric oxide (NO), a molecule synthesised by endothelial cells, plays a major role as a regulator during the onset of immunological and inflammatory reactions (10, 11). It was reported that vascular inflammation is the main pathophysiologic mechanism in BD (10-12). NO may itself regulate endothelial nitric oxide synthase (eNOS) expression and activity and has specifically been shown to play an important negative feedback regulatory role in eNOS, and, therefore, vascular endothelial cell function (13, 14).

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by vascular endothelium and participates in

inflammation, cellular injury, and vascular events. Serum ET-1 levels were higher in BD, especially in the active period (14).

In this study, we investigated the LOX-1 levels in patients with active BD, possible association of LOX-1 with the oxLDL, eNOS, NO, ET-1 levels, and to characterise the differences between patients with BD and those with SLE in terms of these parameters compared with healthy controls.

Materials and methods
The study group included 30 patients in active periods of patients with BD (18 male, 12 female; age:31.93±8.15 year), 22 patients with SLE (3 male, 19 female; age: 36.73±9.55 year) and 30 healthy volunteers (17 male, 13 female; age: 31.10±5.96 year). The diagnosis of BD was made according to the criteria of the International Study Group for Behçet's Disease (15). All SLE patients met at least 4 components of the American College of Rheumatology (ACR) criteria for SLE (16). Additionally, information regarding disease activity (SLE disease activity index: SLEDAI) was collected. For assessment of SLEDAI we used previously validated methodologies. The study was approved by the Ethics Committee of Cerrahpasa Medical Faculty, and all patients and healthy volunteers gave informed consent.

Complete history, physical and laboratory examinations were obtained from all patients. In clinical evaluation, those patients with worsening clinical symptoms at the time of the study, along with having at least three of the major criteria (oral or genital ulcerations; anterior /posterior or panuveitis; papulopustular or pseudofollicular skin lesions; positive pathergy test), were considered to be in the active period of the disease. The number and localisation of affected eye of patients with uveitis attacks (anterior uveitis / posterior uveitis / panuveitis) were identified. All patients were assessed by the same ophthalmologist for eye involvement and received immunosuppression with cyclophosphamide and corticosteroids. Patients with any systemic disease or on any systemic medication (*e.g.* ster-

oids, nonsteroidal anti-inflammatory drugs, vitamins) were excluded.
Also, drugs had been administered more than 24 hours before blood collection. For all persons, clinical parameters including routine biochemical parameters were measured using the standard protocols. Blood samples were collected in EDTA-containing tubes and anticoagulant-free tubes after an overnight fast. Plasma and serum were separated immediately. Three aliquots of 10 samples were prepared for the evaluation of the precision (intra- and inter-assay). Samples were stored at -80°C until analysis.

Measurements of the serum soluble LOX-1 concentrations
Serum sLOX-1 levels were measured by a commercially available enzyme-linked immunosorbent assay kit (Aviscera Bioscience, Santa Clara, USA, lot no. SK00006-01). The coefficients of intra- and inter-assay variation were 5.5% (n=10) and 7.0% (n=10), respectively.

Measurement of plasma MDA-oxLDL concentrations
Plasma MDA-oxLDL concentrations were measured by enzyme-linked immunoassay using commercially available kit (Biomedica, Germany, lot no. BI-20022) in our laboratory. The coefficients of intra- and inter-assay variations were 4.9% (n=10), and 5.8% (n=10), respectively.

Measurement of plasma eNOS activity
Plasma eNOS activity was measured by enzyme-linked immunoassay using commercially available kit (Eastbiopharm, Zheijang, China lot no. CK-E11097) in our laboratory. The coefficients of intra- and inter-assay variations were 5.3% (n=10), and 6.1% (n=10), respectively.

Measurement of plasma endothelin-1 (ET-1) concentrations
Plasma ET-1 concentrations were measured by enzyme-linked immunoassay using commercially available kit (Biomedica, Germany, lot no. BI-20052) in our laboratory. The coefficients of intra- and inter-assay variations were 5.4% (n=10), and 6.2% (n=10), respectively.

Measurement of serum nitric oxide (NOx) concentrations
Serum NOx concentrations were determined by measuring serum NO_2/NO_3 levels using nitrate/nitrite colorimetric assay kit (Cayman Chemical, Michigan USA, lot no. 780001). The coefficients of intra- and inter-assay variations were 3.2% (n=20), and 4.1% (n=20), respectively.

The other biochemical parameters were measured by routine methods with commercial kits. hsCRP measurements were performed by a nephelometric method (BN II nephelometer; Dade Behring Holding GmbH, Liederbach, Germany). Erythrocyte sedimentation rate (ESR) measurements were performed by the Westergren method.

Statistical analysis
Statistical analyses were performed using SPSS 20.0 for Windows. All statistical comparisons were performed using the paired student's *t*-test or unpaired *t*-test. The unpaired *t*-test was also validated using the non-parametric Wilcoxon test. An analysis of variance (ANOVA) was used to compare multiple-group means. $p<0.05$ was considered statistically significant. All data were expressed as the mean ± standard deviation (SD). Pearson's correlation was used for numerical data. To assess the diagnostic accuracy, we performed receiver operating characteristic (ROC) curve analysis. The area under the ROC curve (AUC) was then estimated. $p<0.05$ values were considered to be statistically significant.

Results
Disease manifestations of the patients are summarised in Table I. The demographic characteristics and biochemical parameters of the study subjects are shown in Table II.

ESR and CRP levels were significantly higher in active periods of patients with BD and SLE compared with healthy control ($p<0.001$, both). ESR and CRP levels were significantly increased in patients with SLE compared with active periods of patients with BD ($p<0.001$, $p<0.01$, respectively). Total cholesterol levels were significantly higher in active periods of patients with

Table I. Demographical characteristics and laboratory findings of the BD, SLE and the control group.

	Control (n=30)	SLE (n=22)	Active BD (n=30)
Gender (F/M)	13/17	19/3	12/18
Age (years)	31.10 ± 5.96	36.73 ± 9.55[a]*	31.93 ± 8.15
Disease duration (years)	-	8.77 ± 4.07	5.87 ± 3.69
SLEDAI score	-	8.77 ± 3.68	-
ESR (mm/h)	16.35 ± 9.82	41.23 ± 31.46[a]***	34.33 ± 17.00[b]***
CRP (mg/L)	1.54 ± 0.74	10,78 ± 10,94[a]***	4,73 ± 2,65[b]***
Glucose (mg/dl)	85.74 ± 20.16	93.05 ± 19.38	88.37 ± 14.16
T. Cholesterol (mg/dl)	161.31 ± 18.94	186.64 ± 23.44[a]***	194.20 ± 23.33[b]***
HDL (mg/dl)	50.24 ± 6.74	47.55 ± 9.10	42.27 ± 6.56[b]***,[c]*
LDL (mg/dl)	94.93 ± 19.55	101.00 ± 18.37	118.83 ± 21.54[b]***,[c]**
TG (mg/dl)	92.38 ± 34.08	134.18 ± 35.41[a]***	132.23 ± 33.25[b]***

[a]SLE *vs*. Control; [b]Active BD *vs*. Control; [c]Active BD *vs*. SLE.
*$p<0.05$; **$p<0.01$; ***$p<0.001$.

**Table II.** Endothelial dysfunction parameters in the BD group and the control group.

	Control (n=30)	SLE (n=22)	Active BD (n=30)
eNOS (U/ml)	82.07 ± 50.29	29.09 ± 3.62[a]***	31.67 ± 10.09[b]***
MDA-oxLDL (µg/ml)	13.20 ± 4.61	20.51 ± 5.87[a]***	22.94 ± 5.98[b]***
LOX -1 (pg/ml)	307.96 ± 115.12	456.15 ± 193.60 [a]**	586.00 ± 163.61[b]***,[c]*
ET-1 (fMol/ml)	3.90 ± 0.95	8.13 ± 2.64[a]***	5.86 ± 2.62[b]***,[c]**
NOx (µM)	15.30 ± 4.51	10.80 ± 4.23 [a]***	11.30 ± 4.96[b]**

[a]SLE *vs*. Control; [b]Active BD *vs*. Control; [c]Active BD *vs*. SLE.
*$p<0.05$; **$p<0.01$; ***$p<0.001$.

BD and SLE compared with healthy control ($p<0.001$). Total cholesterol levels were not significant differences between BD and SLE groups. HDL levels were significantly lower in active periods of patients with BD compared with SLE and healthy control ($p<0.05$, $p<0.001$, respectively). HDL levels were not significant differences between SLE and healthy control. LDL levels were significantly higher in active periods of patients with BD compared with SLE and healthy control ($p<0.01$, $p<0.001$, respectively). There were not significant differences between SLE and healthy control. Triglyceride levels were significantly higher in active periods of patients with BD and SLE compared with healthy control ($p<0.001$, both). Triglyceride levels were not significant differences between active periods of patients with BD and SLE.

eNOS activities and NO levels were significantly lower in active periods of patients with BD and SLE compared with healthy control ($p<0.01$, $p<0.001$, respectively). There were not signifi-
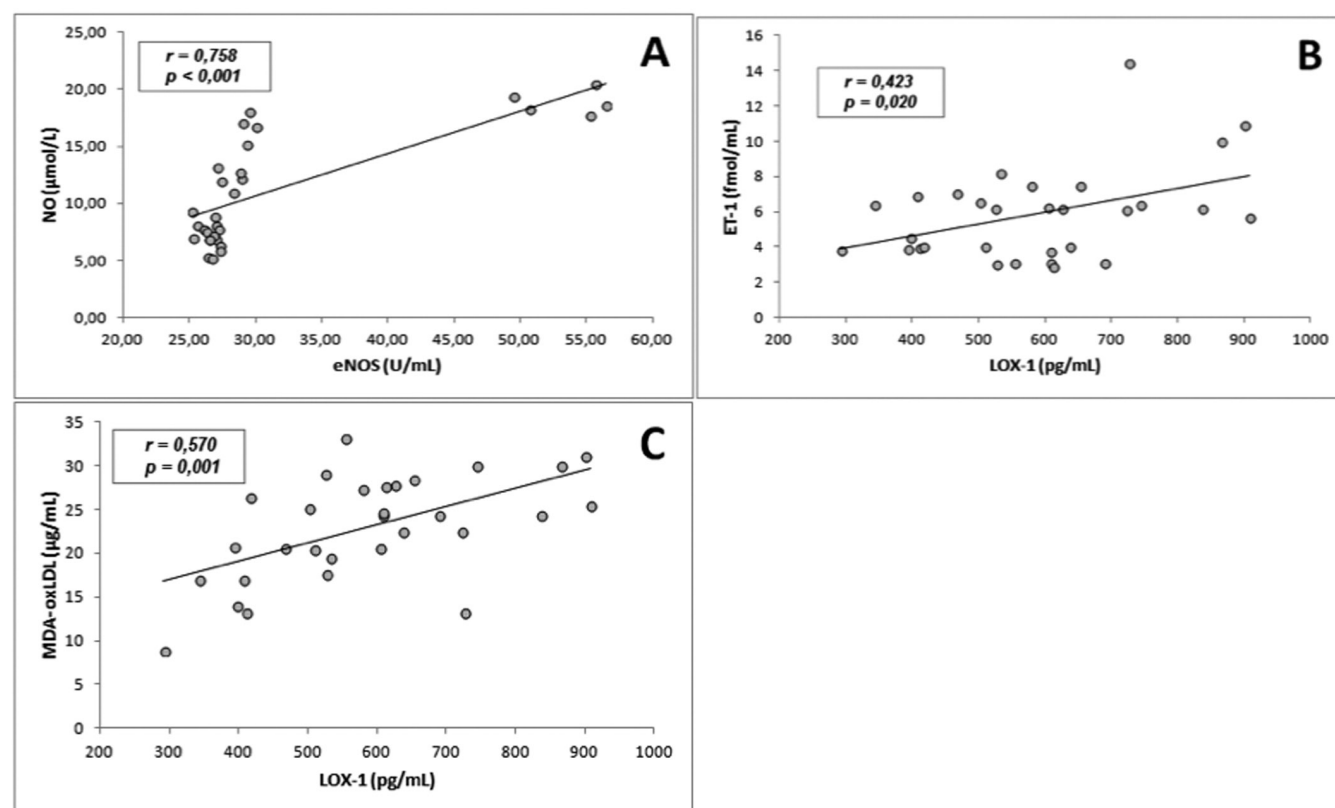
Fig. 1. The relationship between NO with eNOS activities (**A**) and ET-1 with LOX-1 (**B**), MDA-oxLDL with LOX-1 (**C**) in patients with BD.

cant differences between active periods of patients with BD and SLE.

MDA-oxLDL levels were significantly higher in active periods of patients with BD and SLE compared with healthy control ($p<0.001$, $p<0.001$, respectively). There were not significant differences between active periods of patients with BD and SLE.

LOX-1 levels levels were significantly higher in active periods of patients with BD and SLE compared with healthy control ($p<0.001$, $p<0.01$, respectively). LOX-1 levels were significantly higher in active periods of patients with BD compared with SLE ($p<0.05$).

ET-1 levels levels were significantly higher in active periods of patients with BD and SLE compared with healthy control ($p<0.001$, $p<0.01$, respectively). ET-1 levels were significantly higher in patients with SLE compared with healthy control ($p<0.01$).

The LOX-1 levels were positively correlated with the MDA-oxLDL and ET-1 levels in active periods of patients with BD (r=0.570; $p<0,001$; r=0.423; p=0.020, respectively) (Fig. 1 **B**-**C**). There were positive correlations between eNOS and NO in active periods of patients with BD (r=0.758; $p<0.001$) (Fig. 2 **A**).

There were positive correlations between eNOS and NO (r=0.702; $p<0.001$). The sedimantasyon levels were positively correlated with the CRP and LOX-1 levels in SLE patients (r=0.675; $p<0.001$; r=0.544; p=0.009, respectively). The LOX-1 levels were also positively correlated with the levels of CRP in SLE patients (r=0.647; $p<0.001$). A positive correlation was displayed between LOX-1 levels and the SLEDAI in SLE patients.

A comparison of the ROC curves for sensitivity, specificity, AUC, cut-off and asymptotic significance of LOX-1, eNOS, NOx, ET-1, ESR and CRP levels in all subjects are shown in Table III and Figure 2.

Discussion

Despite the evaluation of serum LOX-1 levels in many diseases, there is to our knowledge no data in the literature regarding these parameters in patients with BD and SLE. We have found that

Table III. Sensitivity, specificity, AUC, cut-off and asymptotic significance of LOX-1, eNOS, NOx, ET-1, ESR and CRP levels in all subjects.

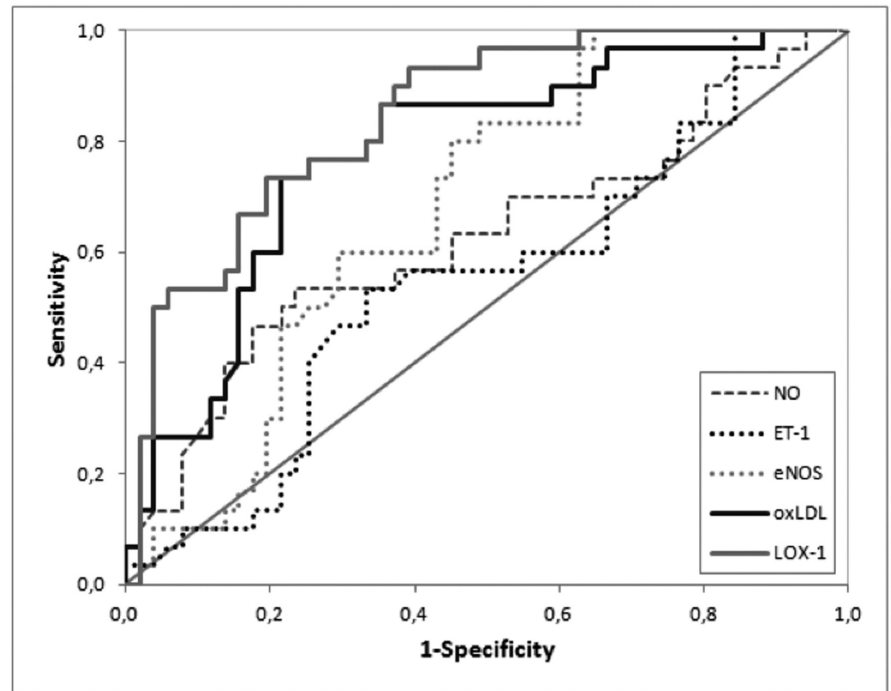|	Sensitivity %	Specifity %	AUC	Cut-off	Asymptotic Sig.
LOX -1 (pg/mL)	93.33	60.78	0.844	> 385.43	<0.001
MDA-oxLDL (µg/ml)	73.33	78.43	0.779	> 20.51	<0.001
eNOS (pg/ml)	100.00	35.29	0.679	≤ 56.45	0.0026
NOx (µM)	53.33	76.47	0.624	≤ 9.41	0.0483
ET-1 (pg/ml)	53.33	66.67	0.547	> 5.99	0.4855
ESR (mm/h)	70.00	62.75	0.680	> 22	0.0046
CRP (mg/L)	70.00	80.39	0.698	> 3.34	0.0015

Fig. 2. ROC curves for NOx, ET-1, eNOS, oxLDL and LOX-1 values in all subjects.

LOX-1 levels were significantly higher in active periods of patients with BD compared with SLE. Its significantly higher levels in BD patients with vascular occlusion, introduces LOX-1 as an important risk factor for atherosclerosis in these patients. Overproduction of LOX-1 could contribute to increase vascular permeability and generate endothelial injury. LOX-1 levels may be useful to monitor as a new marker of vascular endothelial cells activation and of disease activity or therapeutic response in BD. According to ROC analysis, LOX-1 showed only a borderline level of significance (AUC= 0.844, $p<0.001$). Therefore, we believe that this assay may be statistically meaningful, but it may not have any clinical significance in BD. Increased circulating levels of LOX-1 may be one of the

most important factors that are responsible for the development and progression of atherosclerotic lesions.

One of the lipid peroxidation processes occurs in LDL and results in oxidatively modified LDL, known to be the most atherogenic lipoprotein (17). In BD may have a significant role in increased susceptibility of lipoproteins, especially LDL, to oxidative modification. OxLDL plays an important role in the development of atherosclerotic processes and endothelial dysfunction (8, 9). In the two studies made by Orem *et al*. (18, 19) they have detected significantly higher oxLDL autoantibody levels in BD group compared to control group.

High CRP levels have been shown to be associated with endothelial dysfunction. Zhao *et al*. (20) data demon-

strated that CRP enhanced the release of sLOX-1 from activated macrophages *in vitro* and increased circulating sLOX-1 levels *in vivo*. In our study, the sedimantasyon levels were positively correlated with the CRP and LOX-1 levels in SLE patients. The LOX-1 levels were also positively correlated with CRP levels and SLEDAI scores in SLE patients. CRP may act a ligand for LOX-1 and that the interaction of CRP with LOX-1 enhances endothelial inflammation. When the CRP concentration reaches a high level, it may stimulate secretion of inflammatory factors and directly impair endothelial function. When CRP binds with LOX-1 or promotes sLOX-1 release, it may act synergistically with LOX-1 or sLOX-1 to accelerate vascular inflammation and endothelial dysfunction (20).

NO is a powerful vasodilator that it is produced from eNOS. NO has been implicated in the inflammatory reaction in BD. However, the results are controversial. NO has been reported to be higher (21-23) and lower (24-26) in different studies. We found that both eNOS and NO are lower in BD patiens. Decreased eNOS and NOx levels significantly contribute to endothelial dysfunction observed in BD. These contradictory results may be due to genetic variation as NOS polymorphism may affect transcription or function of NO. Also NO may be affected by diet, hydration and renal function (27). But, there were not significant differences between active periods of patients with BD and SLE. There were also positive correlations between eNOS and NOx in our study. Decreased NOx production plays an important role in the pathogenesis of both BD and SLE. Although the mechanism of this impairment is not well understood, a number of studies have shown that the NO synthase pathway and/or the released NO are influenced by oxLDL (28). Vasculitis-associated endothelial dysfunction is recognised as the main cause of decreased/increased NO levels in BD, although its pathogenesis remains to be elucidated. Further studies are needed to determine the cellular and molecular mechanisms by which NO regulates immune cell functions, due to the results are contro-

versial. NOS regime may represent a novel therapeutic approach in the treatment of chronic autoimmune diseases. ET-1 levels are a well known marker of vascular endothelial dysfunction and contribute to ocular pathological manifestations, promoting retinal capillary closure and ischaemia (14). ET-1 may play an important pathogenetic role in the development or progression of vasculitis in Behçet's disease (14, 29-32). Our findings clearly demonstrated that ET-1 levels levels were significantly higher in active periods of patients with BD and SLE compared with healthy control. The LOX-1 levels were also positively correlated with the ET-1 levels in active periods of patients with BD. Er *et al*. (14) demonstrated that serum ET-1 levels were higher in BD, especially in the active period. In addition, ocular BD patients had significantly higher ET-1 levels than non-ocular subjects. The combination of the paracrine effects of ET-1 along with release of NO is likely to have a relevant physiological role in the regulation of blood flow, as ET-1 and NO have opposite effects. ET-1 induces vasoconstriction and thrombosis contributing to pathological manifestations and endothelial damage (32).

BD patients have a high atherogenic potential as marked by the significantly lower HDL, and higher cholesterol and triglicerides concentrations. This study suggests that the abnormal increased of LOX-1 and oxLDL may be important in the pathogenesis of BD, reflect disease activity in a certain extent, especially play an important role in the pathogenesis and early diagnosis of vasculitis. The clinical inference is that measurement, treatment, and monitoring of LOX-1 may be valuable in the management of patients with BD.

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