

RESEARCH ARTICLE

Serum interleukin-1 and interleukin-6 are correlated neither with oxidized low density lipoprotein, nor with low-grade inflammation in patients with type 2 diabetes

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ABSTRACT. *Introduction.* *In vitro* studies have shown that oxidized, low density lipoprotein (ox-LDL) stimulates macrophages to release interleukin-6 (IL-6) and interleukin-1 β (IL-1 β). In this study, we aimed to investigate the correlation between ox-LDL and IL-6 and IL-1 β levels in the peripheral circulation of patients with type 2 diabetes, and normal controls. We measured serum high sensitivity CRP (hs-CRP) levels in order to define basal inflammation in patients and controls. *Methods.* A total of 40 patients with type 2 diabetes, and 40, age, sex, and body mass index (BMI) -matched healthy adults were enrolled in the study. Fasting blood sugar (FBS), lipid profile, creatinine, ox-LDL, IL-6, IL-1 β and hs-CRP levels were measured. *Results.* Patients with type 2 diabetes had higher serum FBS, HbA1C, high density lipoprotein cholesterol, LDL, ox-LDL/LDL ratio and hs-CRP levels than controls. The higher serum ox-LDL/LDL ratio in patients with type 2 diabetes remained significant after multiple adjustments for age, BMI, FBS and HbA1C, (0.65 [0.59-0.71] vs 0.49 [0.41-0.56]; $p < 0.001$) using general linear models. Serum IL-1 β levels were significantly higher in women than in men with type 2 diabetes; this was not the case in controls. Postmenopausal women in patient and control groups had higher serum IL-6 levels than premenopausal women. There was no significant correlation between serum ox-LDL, ox-LDL/LDL ratio, IL-6, IL-1 β and hs-CRP levels in patients with type 2 diabetes. *Conclusion.* Inflammatory cytokines such as IL-1 β and IL-6 lose their discriminatory power with respect to chronic inflammation in patients with type 2 diabetes.

Key words: oxidized low density lipoprotein, interleukin-1 β , interleukin-6, type 2 diabetes

Oxidized, low density lipoprotein (ox-LDL) is a key molecule in the early progression of endothelial dysfunction and atherosclerosis [1]. It is derived from LDL-cholesterol (LDL-C) under oxidative stress, and encompasses many inflammatory, apoptotic and thrombotic pathways in individuals suffering from chronic, oxidative stress, such as diabetes [2]. Diabetes is characterized by increased production of ox-LDL in the peripheral circulation [3, 4]. Furthermore, maintaining an optimized level of LDL in patients with diabetes, does not sufficiently decrease ox-LDL levels in these patients [3].

In vitro studies have shown a role for ox-LDL in the induction of anti-inflammatory and proinflammatory cytokines such as interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) [5, 6]. These cytokines are secreted by T cells and macrophages, and stimulate immune responses and inflammatory pathways [7, 8]. Studies have shown that LDL derivatives induce the secretion of proinflammatory cytokines by activating Toll-like receptor pathways [9-11]. Similarly, Bonaterra *et al.* showed that ox-LDL leads to a

higher expression of inflammatory markers such as TNF- α and IL-1 β in peripheral blood mononuclear cells of hyperlipidemic men [6]. It has been shown that patients with type 2 diabetes have higher concentrations of inflammatory cytokines and a greater number of secretory macrophages, which secrete higher concentrations of interleukins into the peripheral circulation compared with controls [12, 13]. Here, we wanted to study the correlation between ox-LDL and IL-6 and IL-1 β levels in the peripheral circulation of patients with type 2 diabetes and normal controls. We measured serum, high sensitivity CRP (hs-CRP) levels in order to define basal inflammation in patients and controls.

DONORS AND METHODS

We performed a cross-sectional analysis of serum samples from 40 patients with type 2 diabetes attending the diabetes clinic of the Vali Asr hospital, affiliated with the Tehran University of Medical Science, plus 40 controls. Diabetes was diagnosed in accordance with the American

Diabetes Association criteria [14]. Exclusion criteria included treatment with statins, acute or chronic renal failure, glomerulonephritis, congestive heart failure, thyroid disorders, acute infections, stroke, diabetic ketoacidosis, non-ketonic hyperosmolar diabetes, pregnancy and hospital admission within the six months prior to the study. Controls were healthy volunteers from among the patients' families, or hospital staff. The age at menopause, and the time elapsed since the menopause, were also recorded for all patients. None of the participants had overt diabetic complications. Demographic and anthropometric data including age, sex, duration of diabetes, height, weight in light clothing, and blood pressure in a sitting position were recorded. Blood pressure was measured once and then again after approximately five minutes, on average. The body mass index (BMI; kg/m^2) was calculated according to the Quetelet formula. All participants gave their written informed consent before participation. The research was carried out according to the principles of the declaration of Helsinki; the local ethics review committee of Tehran University of Medical Science approved the study protocol.

Blood samples

Blood samples were collected after almost 12 hours of fasting, and serum creatinine, fasting blood sugar (FBS), total cholesterol, triglyceride (TG), high density lipoprotein cholesterol (HDL-C), LDL-C and HbA1C were measured. Glucose measurements (intra-assay coefficient of variants [CV] 2.1%, inter-assay CV 2.6%) were carried out using the glucose oxidase method. Cholesterol, HDL-C, LDL-C and TG were determined using direct enzymatic methods (Parsazmun, Karaj, Iran). HbA1C was estimated using the High-Pressure Liquid Chromatography (HPLC) method (Parsazmun, Karaj, Iran). Creatinine was measured using the calibrated Jaffe method (Parsazmoon, Karaj, Iran). Measurement of ox-LDL- β 2GPI was performed using a commercially-available, sandwich, enzyme-linked immunometric assay (ELISA, Cayman, USA). The intra- and inter-assay coefficients of variation for the assay ranged between 6.4 and 3.4%. Measurement of IL-1 β was performed using a quantitative, sandwich, enzyme-linked immunoassay technique (Human IL-1 β /IL-1F2 immunoassay, Quantikine, USA). The intra- and inter-assay coefficients of variation ranged between 5.5% and 4.3%. Measurement of IL-6 was performed using a quantitative, sandwich, enzyme-linked immunoassay technique (human IL-6 immunoassay, Quantikine, USA). The intra- and inter-assay coefficients of variation ranged between 2.5% and 2.8%. Hs-CRP was assessed using a two-site, enzyme-linked immunosorbent assay (ELISA) (Diagnostic Biochem, London, Ontario, Canada). The sensitivity of the assay was 10 ng/L, with intra- and inter-assay CV of 8% and 10% respectively.

Statistical analysis

The statistical package SPSS 16 for windows (Chicago, Illinois, USA), was used for the analysis. The Kolmogorov-Smirnov test was employed to test the normality of the variables in each group. Variables distributed normally are presented as mean \pm standard deviation (SD) of mean. Variables with skewed distribution are presented as median

[interquintile range]. For comparison of serum hs-CRP, IL-1 β , IL-6, ox-LDL, lipid profile and other variables between the groups, the Mann-Whitney U test and the independent sample T test were employed, as appropriate. The general linear model was employed to compare the ox-LDL/LDL ratios between patients with type 2 diabetes and normal subjects after multiple adjustments for age, BMI, FBS and HbA1C. We then repeated the analysis after stratifying the patients according to gender and menopausal status. The Pearson correlation coefficient was employed to study the correlation of ox-LDL, the ox-LDL/LDL ratio, IL-1, IL-6 and hs-CRP among patients and controls.

RESULTS

Patients with type 2 diabetes had higher serum FBS, HbA1C, HDL-C, LDL-C, ox-LDL/LDL ratios and hs-CRP than controls (*table 1*). The higher serum ox-LDL/LDL ratio in patients with type 2 diabetes remained significant even after multiple adjustment for age, BMI, FBS and HbA1C, (0.65 [0.59-0.71] vs 0.49 [0.41-0.56]; $p < 0.001$) using the general linear model.

Serum IL-1 β levels were significantly higher in women than in men with type 2 diabetes, but there was no such significant gender difference among the controls (*table 2*). Men and women in both the control and patient groups showed no significant differences in serum IL-6, ox-LDL, ox-LDL/LDL ratios or hs-CRP levels (*table 2*). We then stratified women into premenopausal and postmenopausal women. None of the participating women were on hormone replacement therapy or oral contraceptives. Postmenopausal women with diabetes had significantly higher serum IL-6 levels than premenopausal women with diabetes (*table 3*). Likewise, postmenopausal women in the control group also had higher serum IL-6 levels than premenopausal women in the control group (*table 3*).

There was a significant correlation between serum IL-6 and IL-1 β levels in controls ($r = 0.46$; $p < 0.05$). There was no significant correlation between serum ox-LDL, ox-LDL/LDL ratio, IL-6, IL-1 β and hs-CRP levels in patients with type 2 diabetes (*table 4*).

DISCUSSION

In this study, we assessed the hypothesis that ox-LDL might have a role in the inflammatory status of patients with type 2 diabetes. We studied the presence or absence of any possible correlation between ox-LDL, IL-1 β , IL-6 and hs-CRP in patients with type 2 diabetes. Our findings demonstrated that there was no significant correlation between serum hs-CRP, IL-6, IL-1 β and ox-LDL levels in patients with type 2 diabetes. Patients had higher serum FBS, HbA1C, HDL-C, LDL-C, ox-LDL/LDL ratio and hs-CRP levels than controls, although they did not differ as regards serum IL-1 β and IL-6 levels. Postmenopausal women, in both patient and control groups, had higher serum IL-6 levels than premenopausal women.

Given the rarity of clinical studies, there is no definite answer to the question as to if there is any correlation among ox-LDL, IL-1 β or IL-6 *in vivo*. Although

Table 1
Baseline characteristics, lipid profile and cytokine levels in the study groups.

	Patients (n=40)	Control (n=40)	P value
Age (years)	49.18 \pm 9.95	49.15 \pm 9.25	NS
BMI (kg/m ²)	27.77 \pm 4.32	28.03 \pm 3.62	NS
Sex (female, %)	20 (50%)	20 (50%)	NS
Systolic blood pressure (mmHg)	118.3 \pm 14.60	115.25 \pm 10.06	NS
Diastolic blood pressure (mmHg)	75.0 \pm 10.190	76.87 \pm 6.47	NS
Creatinine (mg/dL)	1.02 \pm 0.23	0.92 \pm 0.18	0.02
Duration of disease (years)	5.98 \pm 5.74	-	-
Fasting blood sugar (mg/dL)	156.2 \pm 64.13	89.72 \pm 10.60	<0.001
HbA1C (%)	9.19 \pm 2.69	5.24 \pm 0.61	<0.001
Cholesterol (mg/dL)	198.3 \pm 36.43	231.75 \pm 59.82	<0.01
Triglyceride (mg/dL)	162.62 \pm 60.53	180.45 \pm 110.76	NS
HDL-C (mg/dL)	38.52 \pm 10.55	48.6 \pm 12.89	<0.001
LDL-C (mg/dL)	105.10 \pm 20.01	135.20 \pm 38.08	<0.001
ox-LDL (nm/L)	66.4 [62.3-75.3]	61.5 [57.8-70.8]	NS
ox-LDL/LDL	0.62 [0.60-0.71]	0.49 [0.45-0.55]	<0.001
Interleukin-6 (pg/dL)	2.03 [1.89-3.86]	1.7 [1.75-2.68]	NS
Interleukin-1 β (pg/dL)	1.7 [1.62-2.50]	2.1 [1.8-2.37]	NS
hs-CRP(mg/mL)	2.9 [1.6-2.6]	0.78 [0.7-1.5]	<0.01

Groups are matched for age, BMI and gender.

Variables distributed normally are expressed as mean \pm standard deviation (SD), otherwise median [interquartile range].

BMI: body mass index; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; ox-LDL: oxidized LDL; hs-CRP: high sensitivity CRP; NS: non-significant.

Table 2
Variables studied in patient and control groups, stratified according to gender.

	Patients (n=40)		Controls (n=40)	
	Women (n=20)	Men (n=20)	Women (n=20)	Men (n=20)
Age (years)	49.35 \pm 9.27	49.0 \pm 10.83	49.65 \pm 9.05	48.65 \pm 9.71
BMI (kg/m ²)	28.92 \pm 4.88	26.58 \pm 3.415	29.04 \pm 4.21	27.02 \pm 2.65
Systolic blood pressure (mmHg)	118.75 \pm 15.88	118.0 \pm 13.61	112.50 \pm 11.97	118.0 \pm 6.95
Diastolic blood pressure (mmhg)	75.75 \pm 11.38	74.25 \pm 9.07	76.25 \pm 6.66	77.5 \pm 6.38
FBS (mg/dL)	155.450 \pm 68.59	157.0 \pm 61.12	88.50 \pm 9.28	90.95 \pm 11.88
HBA1C (%)	8.47 \pm 2.26	9.9 \pm 2.90	5.21 \pm 0.60	5.28 \pm 0.63
Cholesterol (mg/dL)	198.85 \pm 40.5	197.750 \pm 32.90	216.15 \pm 53.84	247.35 \pm 62.77
LDL-C (mg/dL)	103.15 \pm 19.98	107.05 \pm 20.36	127.90 \pm 39.38	142.50 \pm 36.07
HDL-C (mg/dL)	39.3 \pm 12.21	37.75 \pm 8.84	51.45 \pm 10.67	45.75 \pm 14.54
Triglyceride (mg/dL)	165.95 \pm 60.17	159.30 \pm 62.26	139.65 \pm 56.37	221.25 \pm 136.026*
Creatinine (mg/dL)	0.935 \pm 0.20	1.120 \pm 0.23*	0.90 \pm 0.159	0.94 \pm 0.21
ox-LDL (nm/L)	61.4 [87.4-77.6]	69.5 [61.4-78.4]	49.8 [49.7-67.8]	66.2 [60.4-79.4]
Interleukin-6 (pg/dL)	2.3 [1.9-3.8]	1.6 [0.8-4.7]	2.1 [1.58-3.3]	1.7 [1.5-2.4]
Interleukin-1 β (pg/dL)	2.4 [1.8-3.2]	1.3 [1.1-1.97]*	1.7 [1.6-2.7]	2.2 [2.4-3.2]
Ox-LDL/LDL	0.6 [0.58-0.72]	0.62 [0.57-0.74]	0.4 [0.41-0.57]	0.49 [0.45-0.58]
hs-CRP(mg/mL)	2.0 [1.7-3.2]	1.2 [0.9-2.5]	1.0 [0.7-1.8]	0.6 [0.6-1.6]

Variables distributed normally are expressed as mean \pm standard deviation (SD), otherwise median [interquartile range]. Normally distributed variables are compared using Student's t test, and variables deviating from normal distribution are compared using the Mann-Whitney U test for comparison of men and women.* p<0.05, ** p<0.01, when comparing women with men in diabetes and control groups.

previous studies have shown a causal relationship between ox-LDL and IL-1 β and IL-6 in patients with unstable angina [15], coronary artery disease [15] and mobility disability [16], our study shows that there is no such cor-

relation between ox-LDL, and IL-1 β and IL-6, in patients with type 2 diabetes. This finding is also consistent with the study by Lu *et al.* which showed that ascorbic acid supplementation did not reduce serum IL-1 β , IL-6 and ox-

Table 3
Variables studied in women, stratified according to menopausal status.

	Women with type 2 diabetes (n=20)		Women in control group (n=20)	
	Postmenopausal (n=9)	Premenopausal (n=11)	Postmenopausal (n=9)	Premenopausal (n=11)
Age (years)	57.78±4.842***	42.45±5.336	58.11±3.06***	42.73±5.42
BMI (kg/m ²)	30.49±3.41	27.63±5.64	30.89±2.48	27.53±4.82
Systolic blood pressure (mmHg)	123.8±13.64	114.5±16.91	118.3±8.66*	107.7±12.52
Diastolic blood pressure (mmHg)	80.5±9.50	71.8±11.67	78.8±3.33	74.0±8.00
FBS (mg/dL)	164.7±65.44	147.8±73.28	89.3±8.78	87.81±10.04
HBA1C (%)	7.8±1.9	9.0±2.44	5.1±0.78	5.2±0.40
Cholesterol (mg/dL)	216.33±32.46	184.5±42.13	241.4±57.79*	195.45±42.29
LDL-C (mg/dL)	108.5±20.40	98.7±19.43	143.22±39.60	115.36±36.16
HDL-C (mg/dL)	43.6±15.39	35.72±7.91	50.66±10.78	52.0±11.06
Triglyceride (mg/dL)	178.8±49.92	155.36±67.91	159.4±54.59	123.45±54.89
ox-LDL (nm/L)	60 [47.8-88.7]	66 [54.9-78.9]	67 [50.4-80.5]*	48.2 [40.6-64.1]
Interleukin-6 (pg/dL)	2.7 [1.7-5.1]*	1.8 [1.4-2.1]	2.7 [1.1-5.15]**	1.4 [1.1-1.8]
Interleukin-1β (pg/dL)	1.8 [1.3-3.4]	2.5 [1.6-3.8]	1.6 [0.4-5.6]	2.4 [1.4-3.4]
Ox-LDL/LDL	0.5 [0.4-0.7]	0.7 [0.6-0.7]	0.4 [0.3-0.6]	0.4 [0.3-0.5]
hs-CRP(mg/mL)	3.37 [1.9-3.6]	1.7 [1.0-3.4]	0.8 [0.4-1.3]	1.6 [0.7-2.7]

Variables distributed normally are expressed as mean ± standard deviation (SD), otherwise median [interquartile range]. Normally distributed variables are compared using Student's t test, and variables deviating from normal distribution are compared using the Mann-Whitney U test for comparison of men and women. * p<0.05, ** p<0.01, when comparing premenopausal and postmenopausal women in diabetes and control groups.

Table 4
Correlation coefficients among serum interleukin-6, interleukin-1β, ox-LDL, ox-LDL/LDL ratio and hs-CRP in patients with type 2 diabetes and controls.

		Interleukin-6 (pg/dL)	Interleukin-1β (pg/dL)	ox-LDL (nm/L)	hs-CRP (mg/mL)
Patients (n=40)	Interleukin-6 (pg/dL)	1	0.081	0.030	-0.08
	Interleukin-1β (pg/dL)	0.081	1	-0.072	0.1
	ox-LDL (nm/L)	0.030	-0.072	1	-0.17
	ox-LDL/LDL ratio	0.061	0.079	0.778**	-0.16
Controls(n=40)	Interleukin-6 (pg/dL)	1	0.361*	0.016	0.16
	Interleukin-1β (pg/dL)	0.461*	1	0.217	0.18
	ox-LDL (nm/L)	0.016	0.217	1	0.04
	ox-LDL/LDL ratio	0.016	0.026	0.538**	0.06

* p<0.05, ** p<0.01, *** p<0.001.

LDL in 17 patients with type 2 diabetes [17]. Furthermore, they showed that there was no correlation between serum IL-1β, IL-6 and ox-LDL. Similarly, Doo *et al.* suggested that anti-ox-LDL was not significantly correlated with IL-6 [18]. Guldien *et al.* showed that mean ox-LDL levels were higher in type 2 diabetic patients with large vessel disease, although mean IL-6 levels were higher in type 2 diabetic patients with small vessel disease [19]. Similar studies have shown that the ox-LDL concentration does not correlate with inflammatory markers such as IL-1β, IL-6 and TNF-α in postmenopausal women with metabolic syndrome [20, 21].

We showed that there was no significant difference between patients with type 2 diabetes and normal controls as regards serum IL-6 and IL-1β levels. Although several studies confirm our findings [22-25], others suggest the contrary [26, 27]. Saremi *et al.* showed that despite a generally higher level of inflammation in patients with type 2 diabetes, serum IL-6 levels predict only coronary artery calcification in these patients [28]. Park *et al.* showed

that there was no significant difference in serum IL-1β and IL-6 levels between postmenopausal women with and without metabolic syndrome. Hence, one would argue that these findings may be confounded by the hormonal status of the participants. Interestingly, and in agreement with previous studies, we showed that postmenopausal women had higher serum IL-6 levels than premenopausal women, in both patient and control groups [29, 30]. The numbers of premenopausal and postmenopausal women in patient and control groups were also similar. Hence, we believe that the menopausal status of participants does not confound our findings.

There are at least three reasons that could explain our results. It is suggested that some of the relationships observed in *in vitro* studies disappear *in vivo*. It is known that intracellular O₂ levels are low for most cells in the human body, which diminishes oxidative stress. On the other hand, most animal cells are cultured under 95% air and 5%CO₂, which is grossly hyperoxic and increases oxidative stress [31]. Moreover, cell culture media are

always deficient in antioxidants, and have free ions, which accelerate the development of oxidative stress [31]. This may explain why the casual relationship observed in *in vitro* studies disappears in *in vivo* situations.

Secondly, immune complexes, formed from anti ox-LDL and LDL, stimulate macrophages to release markedly high amounts of TNF- α and IL-1 β . These markers induce smooth muscle cell proliferation and increased cell adhesion, which play important roles in the pathogenesis of atherosclerosis [32]. Interestingly, Goncalves *et al.* showed that simvastatin treatment induces an increase in production of auto-antibodies against ox-LDL [33]. Statin treatment was an exclusion criterion in our study. We think this might have confounded previous reports.

Thirdly, these biomarkers are synthesized via different pathways. This diversity in the nature and origin of these inflammatory markers and cytokines in patients with type 2 diabetes may be another reason. Interestingly, serum IL-1 β and IL-6 levels correlated significantly with each other in controls, although there was no such significant correlation in patients. IL-1 β is a proinflammatory cytokine that is mainly secreted as a result of intracellular inflammation [34, 35]. It binds to proteins and is associated with pancreatic β cell secretory function, increases in hexokinase activity and hexokinase II isoform abundance in mesangial cells [36-38]. On the other hand, the anti-inflammatory IL-6 cytokine, stimulates the appearance of anti-inflammatory cytokines such as IL-1 receptor antagonist and IL-10, and inhibits the production of proinflammatory cytokines such as TNF- α [39]. IL-6 is an anti-inflammatory cytokine and an acute phase reactant, whereas diabetes is associated with chronic inflammation. So, IL-6 loses its discriminatory power with respect to the chronic inflammation of disease, among patients with conditions in which inflammatory markers are increased such as in type 2a diabetes.

We also showed that women with type 2 diabetes had significantly higher serum IL-1 β levels compared to men with diabetes, and the women and men in the control group. Recent studies have shown the role of gender in the mechanisms regulating oxidant/antioxidant pathways in patients with diabetes [40-43]. Women with diabetes have been shown to secrete higher hs-CRP levels than men [40]. We have previously shown that serum HSP 70 levels are significantly higher in women with type 2 diabetes [44]. IL-1 β increases in response to intracellular inflammation [34, 35], when ox-LDL is usually formed enzymatically, within the circulation [45]. However, the titration of IL-1 β alone does not provide a clear view of the inflammatory cytokine balance since levels of the inhibitory cytokine IL-1 receptor antagonist have to be evaluated simultaneously [46]. IL-1 receptor antagonist is a member of the interleukin-1 cytokine family, which inhibits the activities of IL-1 β and modulates a variety of interleukin-1-related immune and inflammatory responses [46]. Many studies have shown higher serum IL-1 receptor antagonist levels, independent of the degree of adiposity and glucose control, in women with type 2 diabetes and metabolic syndrome [40-43]. So, without measuring serum IL-1 receptor antagonist, we cannot conclude that intracellular inflammation is more severe in women with type 2 diabetes. Whether accelerated inflammation in women with type 2

diabetes is mediated by the pathways that are associated with IL-1 β secretion has to be tested by studies measuring IL-1 receptor antagonist simultaneously.

The principal limitation of the present study is its cross-sectional nature, which precludes the determination of the direction of causality. Furthermore, we did not measure serum IL-1 receptor antagonist or TNF- α levels in these patients. TNF- α is one of the important cytokines indicative of the level of basal inflammation. These are interesting topics that could be covered in future studies. On the other hand, we took advantage of a relatively large sample size and a close similarity between groups as regards most of the confounding variables. In conclusion, we showed that ox-LDL does not have any significant correlation with IL-1 β or IL-6. Furthermore, we showed that IL-1 β and IL-6 levels are not increased in patients with type 2 diabetes. Future, prospective studies may elucidate the role of ox-LDL in the induction of systemic inflammation in patients with type 2 diabetes.

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