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Circulating Oxidized Low-Density Lipoprotein Levels Independently Predict 10-Year Progression of Subclinical Carotid Atherosclerosis: A Community-Based Cohort Study

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Aims: To investigate the association between circulating oxidized low-density lipoprotein (ox-LDL) levels and progression of subclinical atherosclerosis and to examine whether this link is independent of other low-density lipoprotein (LDL)-related parameters.

Methods: Totally, 804 subjects who were free of cardiovascular disease at baseline completed risk factor surveys and carotid ultrasound measurements in 2002 and 2012. Modified Poisson regression was performed to examine the association between baseline serum ox-LDL levels and the 10-year risk of progression of carotid atherosclerosis which was defined as the development of at least one new plaque in a previously plaque-free carotid segment at re-examination.

Results: The mean age of the subjects was 58.6±7.7 years at baseline and 43.3% were men. A total of 504 (62.7%) subjects had carotid plaque progression at re-examination. Subjects in the intermediate and highest tertiles of ox-LDL had a significantly higher adjusted risk of atherosclerosis progression than those in the lowest tertile [relative risk (95% confidence interval) 1.17 (1.01−1.34) for the intermediate tertile and 1.23 (1.07−1.42) for the highest tertile]. This association was independent of baseline levels of LDL-C, total LDL particle number, and small LDL particle number.

Conclusion: This study demonstrates that serum ox-LDL levels predict 10-year progression of subclinical atherosclerosis. Moreover, this effect is independent of the cholesterol content, the number, and the size of LDL particles.

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Key words: Atherosclerosis, Carotid stenosis, Cohort study, Oxidized low-density lipoprotein, Risk factors

Introduction

Oxidized low-density lipoprotein (ox-LDL) is the oxidatively modified form of low-density lipoprotein (LDL), a causal risk factor of atherosclerosis[1]. Different from native LDL, ox-LDL can be identified and internalized by scavenger receptors, which are not down-regulated by elevated intracellular cholesterol levels, to induce cholesterol accumulation inside macrophages and foam cell formation. Subsequently, development and progression of atherosclerosis occur[1]. In addition to the accumulation of cholesterol as a substrate, ox-LDL can induce a wide range of pro-atherogenic bioactive effects. These effects include endothelial dysfunction and activation, vascular cellular proliferation, and thrombosis formation, partly through uptake of ox-LDL by the lectin-like ox-LDL receptor (LOX-1) in vessel cells[2, 3].

Since the discovery of ox-LDL three decades ago, many observational studies have focused on the asso-

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ciation between circulating ox-LDL and atherosclerotic cardiovascular disease. Our previous meta-analysis of prospective studies reported that increased circulating ox-LDL levels were associated with clinical atherosclerotic cardiovascular disease[4]. However, clinical atherosclerotic cardiovascular disease is the end stage of atherosclerosis. Evidence from prospective studies investigating whether circulating ox-LDL levels affected atherosclerosis prior to clinical events, i.e., subclinical atherosclerosis is limited and inconclusive[5, 6]. Additionally, whether the pro-atherogenic effects of ox-LDL are specifically due to modification of LDL, or are merely driven by the effect of LDL cholesterol (LDL-C) or LDL particles remains unclear. Therefore, we aimed to investigate the association between baseline circulating ox-LDL levels and 10-year progression of carotid atherosclerosis, and to examine whether this association is independent of other LDL-related parameters in asymptomatic individuals from a community-based cohort study.

Material and Methods

Study Subjects

Study subjects were recruited from the Chinese Multi-Provincial Cohort Study-Beijing Project, which is a prospective general population-based cohort study that focused on investigating progression and determinants of carotid atherosclerosis[7, 8]. A total of 1324 subjects, aged 45−74 years, completed examinations on demographic characteristics, traditional cardiovascular risk factors, and carotid ultrasound examination in 2002. After excluding those with clinical cardiovascular disease and those without blood samples at baseline, 1116 subjects were invited to be re-examined for risk factors and carotid ultrasound measurements in 2012. Finally, 804 subjects with complete information in both surveys and who were free of cardiovascular disease at baseline were eligible for the current study (**Fig. 1**). During the 10-year follow-up period, 28 cardiovascular events occurred. Written informed consent was obtained from all subjects. The protocol was approved by the Ethics Committee of Beijing An Zhen Hospital, Capital Medical University.

Risk Factor Survey

A standardized questionnaire modified according to the WHO-MONICA protocol for risk factor survey was used to collect information in both surveys for demographic characteristics, personal medical history, and medical therapy[9]. Height, weight, and blood pressure levels were measured during physical examinations. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Hypertension was defined as a systolic blood pressure (SBP) ≥140 mmHg and/or a diastolic blood pressure (DBP) ≥90 mmHg, or use of anti-hypertension drugs in the past 2 weeks[10]. Type 2 diabetes was considered if fasting blood glucose (FBG) levels were ≥7.0 mmol/L or with a clinical diagnosis[11]. Information of statin use was gathered in 2007. Details of survey methods and definition of risk factors were described previously[7, 12].

Laboratory Measurements

Overnight fasting venous blood samples were collected for laboratory measurements. Total cholesterol (TC), triglyceride (TG), LDL-C, high-density lipoprotein cholesterol (HDL-C), and FBG levels were measured by enzymatic methods (TC, TG, and FBG) or homogeneous assay (LDL-C and HDL-C). Non-HDL cholesterol (non-HDL-C) level was calculated using TC minus HDL-C. Measurements were made on fresh samples collected on the day of the survey. The remaining samples were stored at −80℃ without repeated freeze-thaw cycles to minimize any degradation. The number and size of plasma LDL particles were measured in 2013 using a nuclear magnetic resonance spectroscopy assay at LipoScience (Raleigh, NC)[13]. Serum lipoprotein (a) (Lp(a)) and high-sensitivity C-reactive protein (hs-CRP) levels were measured in 2015 on an automatic biochemical analyzer (Hitachi 7180, Hitachi, Japan) using a latex-enhanced immunoturbidimetric assay (Denka Seiken, Ltd.). Serum ox-LDL levels were measured in 2015 by ELISA kit (Mercodia AB, Uppsala, Sweden) using murine monoclonal antibody 4E6. Pre-study validation was performed by determining 22 low-level and 22 high-level control samples in duplicate on consecutive days. In formal tests, low-level and high-level samples were randomly added in each assay in duplicate. The mean duplicate coefficient of variation was 6.22% for low-level control samples and 5.17% for high-level control samples.

Carotid Atherosclerosis Examination

A standard measurement protocol was used for baseline and follow-up carotid B-mode ultrasound examinations. Briefly, carotid ultrasonography was performed in six different carotid segments (far and near walls of bilateral common carotid arteries, bifurcations, and internal carotid arteries). The presence of plaques was defined as a focal region with intimal-medial thickness (IMT) ≥1.5 mm or a focal structure that encroached into the arterial lumen measuring at least 0.5 mm or 50% of the surrounding IMT[14]. Progression of carotid plaques was defined as development of at least one new plaque in a previously plaque-free carotid segment at re-examination[15, 16]. The results of reproducibility

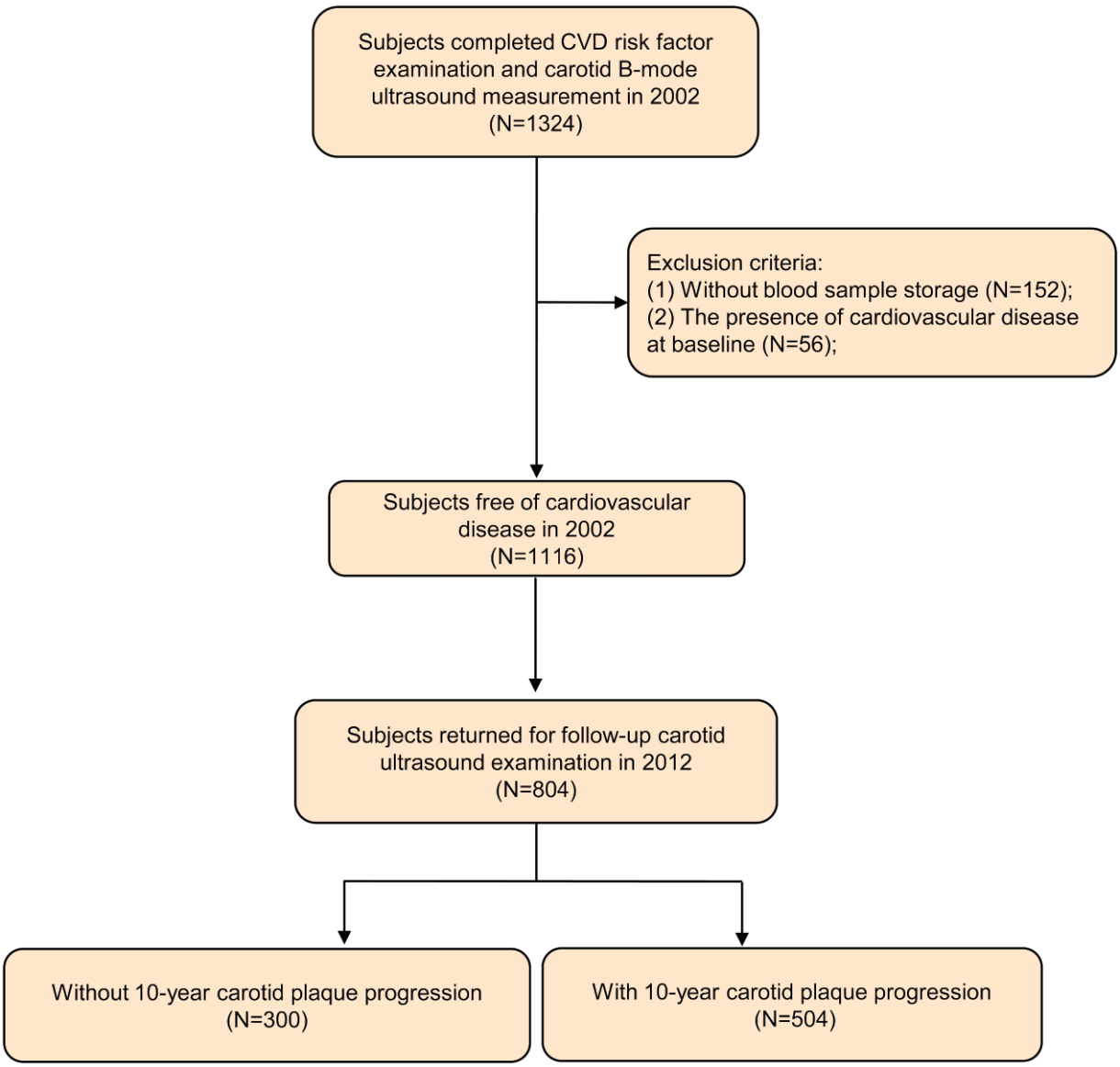

Fig. 1. Flowchart for selection of study subjects.

at baseline were described previously[7]. Image sequences of subjects with carotid plaques were randomly selected to be measured twice by all three observers (2 weeks apart) to assess the intra- and interobserver reproducibility of carotid IMT at re-examination. The average intraclass correlation coefficient for interobserver reproducibility of carotid IMT was 0.937 (95% confidence interval [CI]: 0.843−0.975), and the minimum intraclass correlation coefficient for intraobserver agreement was 0.928 (95% CI: 0.810−0.972).

Statistical Analysis

Continuous variables are expressed as mean ± standard deviation and were compared by one-way analysis of variance (ANOVA) if normally distributed. Continuous variables in skewed distribution are expressed as the median (interquartile range) and were compared by the Kruskal−Wallis test. Categorical variables are expressed as proportions (%) and were compared by the χ^2 test. Correlations between serum ox-LDL levels and baseline lipid parameters were evaluated using the Spearman correlation method and further adjusted for age and sex in a partial correlation analysis.

The 10-year progression rates of carotid plaques were compared by tertiles of baseline serum ox-LDL levels (cutoff values: <41.0, 41.0−54.4, ≥ 54.5 U/L) using the χ^2 test. Bonferroni correction was adopted in multiple comparisons. Modified Poisson regression[13]

Table 1. Baseline characteristics of subjects by oxidized LDL tertiles

Baseline characteristics	Tertiles of oxidized LDL			*P*-value
	Tertile 1 (*N*=268)	Tertile 2 (*N*=268)	Tertile 3 (*N*=268)	
Age (years)	57.8±7.9	58.8±7.6	59.3±7.5	0.091
Male (%)	113 (42.2)	119 (44.4)	116 (43.3)	0.872
Current smoker (%)	26 (9.7)	22 (8.2)	28 (10.4)	0.664
Body mass index (kg/m^2)	24.6±3.2	24.9±3.3	25.3±2.9	0.053
Type 2 diabetes (%)	18 (6.7)	13 (4.9)	27 (10.1)	0.060
Fasting blood glucose (mmol/L)	4.8±0.8	4.8±0.7	5.1±1.4	0.001
Glucose-lowering drugs (%)	10 (3.7)	5 (1.9)	11 (4.1)	0.292
Hypertension (%)	110 (41.0)	119 (44.4)	139 (51.9)	0.036
Use of anti-hypertensive drugs (%)	61 (22.8)	79 (29.5)	90 (33.6)	0.020
Systolic blood pressure (mmHg)	125.3±16.9	127.1±17.0	130.5±18.0	0.002
Diastolic blood pressure (mmHg)	78.9±9.6	80.2±9.8	82.2±10.1	<0.001
Total cholesterol (mmol/L)	5.0±0.9	5.5±0.8	6.2±1.0	<0.001
LDL cholesterol (mmol/L)	2.9±0.7	3.3±0.6	3.8±0.8	<0.001
HDL cholesterol (mmol/L)	1.4±0.3	1.4±0.3	1.3±0.3	0.002
non-HDL cholesterol (mmol/L)	3.5±0.8	4.1±0.7	4.8±0.9	<0.001
Triglyceride (mmol/L)	1.1 (0.8-1.5)	1.2 (0.9-1.7)	1.6 (1.2-2.3)	<0.001
Lipoprotein (a) (mg/dl)	8.8 (5.4-14.4)	10.6 (6.5-18.5)	11.3 (7.5-18.7)	<0.001
LDL particle number (nmol/L)				
Total	922.2±235.3	1059.2±188.4	1263.1±259.8	<0.001
Large	222.5 (124.0-342.8)	270.5 (157.3-386.5)	261.0 (109.5-386.0)	0.043
Small	470.5 (340.3-626.3)	549.5 (409.0-762.3)	764.0 (521.8-1000.0)	<0.001
LDL particle size (nm)	20.7±0.6	20.6±0.6	20.5±0.6	<0.001
hs-CRP (mg/L)	0.9 (0.5-1.9)	1.2 (0.5-2.2)	1.1 (0.7-2.3)	0.003
Use of statins (%)	10 (3.7)	8 (3.0)	31 (11.6)	<0.001
Oxidized LDL (U/L)	32.7 (25.6-37.4)	47.1 (44.3-51.2)	64.5 (58.4-74.9)	<0.001

LDL, low density lipoprotein; HDL, high density lipoprotein; hs-CRP, high-sensitivity C-reactive protein.

was performed to calculate the relative risks (RRs) and 95% CIs of the baseline serum ox-LDL tertiles for 10-year risk of progression of carotid atherosclerosis after adjusting for age, sex, current smoker, type 2 diabetes, SBP, use of anti-hypertension drugs, hs-CRP, TG, HDL-C, and use of statins.

We further examined the association between baseline serum ox-LDL levels and progression of carotid atherosclerosis independent of LDL-related parameters, including LDL-C, total LDL particle number, and small LDL particle number. To avoid collinearity of ox-LDL levels with these three LDL-related parameters, we performed 2×2 combined group analyses. First, variables were categorized by median levels. Subsequently, four combined groups were generated between ox-LDL and LDL-C (i.e., low ox-LDL and low LDL-C, low ox-LDL and high LDL-C, high ox-LDL and low LDL-C, and high ox-LDL and high LDL-C). Combinations of ox-LDL with total LDL particle number and small LDL particle number were generated similarly. RRs for the 10-year risk of plaque progression and 95% CIs were calculated for combinations of ox-LDL levels with LDL-C, total LDL particle number and small LDL particle number in model 1, 2, and 3, respectively. The groups with low levels of ox-LDL and LDL-related parameters were used as references. Age, sex, current smoker, type 2 diabetes, SBP, use of anti-hypertension drugs, hs-CRP, TG, HDL-C, and use of statins were adjusted in models 1 and model 2. Because of strong correlations between TG, HDL-C, and small LDL particle number levels, the first two covariates were not further adjusted in model 3. Sensitivity analyses were performed after excluding subjects on statin treatment at baseline (n=49), with any carotid plaque at baseline (n=156), or having cardiovascular diseases during the 10-year follow-up period (n=28), respectively. Additionally, the ratio of ox-LDL and LDL particle number (units of ox-LDL per nmol of LDL particle number) was calculated to represent the proportion of oxidized LDL in total LDL particles. RRs and 95% CIs of 10-year progression of carotid atherosclerosis across tertiles of the baseline ox-LDL/LDL particle number

Table 2. Spearman correlations between baseline oxidized LDL levels and lipid parameters.

Baseline characteristics	R	*P*-value	Partial R †	*P*-value
Total cholesterol (mmol/L)	0.508	<0.001	0.444	<0.001
LDL cholesterol (mmol/L)	0.491	<0.001	0.413	<0.001
HDL cholesterol (mmol/L)	−0.113	<0.001	−0.045	0.202
non-HDL cholesterol (mmol/L)	0.564	<0.001	0.471	<0.001
Triglyceride (mmol/L)	0.337	<0.001	0.213	<0.001
Lipoprotein (a) (mg/dl)	0.151	<0.001	0.104	0.003
LDL particle number (nmol/L)				
Total	0.531	<0.001	0.450	<0.001
Large	0.036	0.303	0.041	0.249
Small	0.389	<0.001	0.322	<0.001
LDL particle size (nm)	−0.162	<0.001	−0.098	0.006

LDL, low density lipoprotein; HDL, high density lipoprotein.
† Spearman partial correlations after adjustment for age and sex.

ratio were also calculated after adjustment for age, sex, current smoker, type 2 diabetes, SBP, anti-hypertensive drug use, hs-CRP, TG, HDL-C, and use of statins. Considering that elevated blood pressure is an important risk factor for carotid IMT progression[17], we also generated 2×2 combinations between ox-LDL and blood pressure levels to evaluate the effect of ox-LDL independent of blood pressure levels. Statistical significance was set at a *P*-value <0.05 (two-sided), except for Bonferroni correction. All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC).

Results

Baseline Characteristics

The mean age of the subjects was 58.6±7.7 years at baseline and 43.3% were men. Ox-LDL levels had a skewed distribution and were similar between sexes, with median (interquartile range) values of 48.1 U/L (37.2−57.9 U/L) for men and 46.5 U/L (37.4−58.8 U/L) for women (*P*=0.675). Baseline characteristics of the subjects according to ox-LDL tertiles are shown in **Table 1**. Levels of FBG, blood pressure, TG, TC, LDL-C, non-HDL-C, Lp(a), hs-CRP, and LDL particle numbers were significantly higher, whereas HDL-C levels and LDL particle size were lower in subjects with higher ox-LDL levels. Subjects with higher baseline ox-LDL levels were more likely to have hypertension and higher rates of anti-hypertensive drug use and statin use.

Correlations between Baseline Ox-LDL Levels and Lipid Parameters

Ox-LDL levels were correlated with the majority of baseline lipid parameters, except for large LDL particle number. Briefly, ox-LDL levels were most closely correlated with non-HDL-C levels, followed by the total LDL particle number, TC levels, LDL-C levels, small LDL particle number, and TG levels. Ox-LDL levels were also positively correlated with Lp(a) and negatively correlated with LDL particle size and HDL-C levels, but these correlations were rather weak. Similar correlations were shown after adjustment for age and sex, except that the correlation with HDL-C disappeared (**Table 2**).

Association between Ox-LDL Levels and the 10-Year Risk of the Progression of Carotid Atherosclerosis

A total of 504 (62.7%) subjects developed at least one new plaque in a previously plaque-free carotid segment at re-examination. Compared with subjects with the lowest baseline serum ox-LDL tertile, there was a significant increase in the 10-year risk of progression of carotid atherosclerosis in subjects in the intermediate and the highest tertiles of ox-LDL (**Fig. 2**). Similar results were shown after adjustment for other risk factors in multivariate regression analysis (**Table 3**).

Furthermore, combined group analyses were performed to investigate whether ox-LDL levels are associated with 10-year progression of atherosclerosis independent of other LDL-related parameters (**Table 4**). Results from models 1 to 3 showed that after adjustment for other covariates, the 10-year risk of progression of atherosclerosis significantly increased in subjects with higher ox-LDL levels, regardless of whether baseline LDL-C levels, total LDL particle number, and small LDL particle number levels were high or low. These effects of ox-LDL on 10-year atherosclerosis progression independent of other LDL-related parameters were not substantially changed in sensitivity analyses, although some of these effects were borderline significant due to smaller sample size (**Table 5**). Moreover,

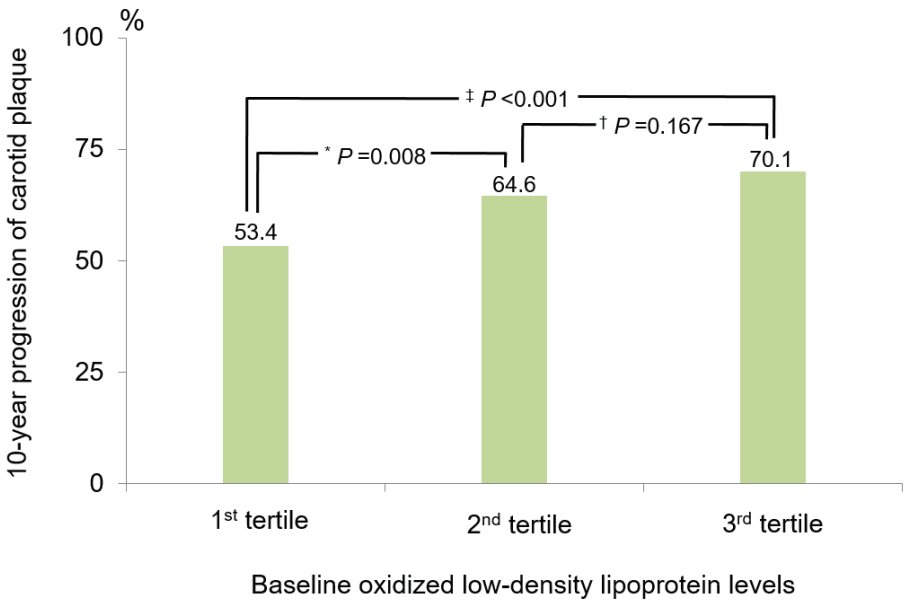

Fig. 2. Ten-year progression of carotid atherosclerosis by baseline tertiles of oxidized low-density lipoprotein. *1st tertile vs. 2nd tertile, †2nd tertile vs. 3rd tertile, ‡1st tertile vs. 3rd tertile.

Table 3. Relative risks (RRs) and 95% confidence intervals (CIs) of 10-year risk of progression of carotid atherosclerosis associated with baseline oxidized LDL tertiles.

Variables	RR (95%CI)	*P*-value
Oxidized- LDL tertiles †		
T1	Reference	
T2	1.17 (1.01-1.34)	0.031
T3	1.23 (1.07-1.42)	0.003
Age (years)	1.02 (1.01-1.03)	<0.001
Male	0.97 (0.86-1.10)	0.644
Current smoker	1.19 (1.00-1.41)	0.052
Type 2 diabetes	1.07 (0.90-1.29)	0.435
Systolic blood pressure (mmHg)	1.00 (1.00-1.01)	0.044
Use of anti-hypertensive drugs	1.02 (0.92-1.15)	0.669
hs-CRP (mg/L)	1.00 (0.99-1.01)	0.819
Triglyceride (mg/dl)	1.00 (1.00-1.00)	0.680
HDL cholesterol (mg/dl)	0.99 (0.99-1.00)	0.027
Use of statins	0.96 (0.79-1.18)	0.721

LDL, low density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; HDL, high density lipoprotein.
† T1 (the lowest tertile): oxidized LDL <41.0 U/L; T2 (the intermediate tertile): 41.0 ≤oxidized LDL L <54.5 U/L; T3 (the highest tertile): oxidized LDL ≥54.5 U/L.

the ox-LDL/total LDL particle number ratio was calculated to represent the proportion of oxidized LDL particles in the total number of LDL particles. After adjustment for covariates, the 10-year risk of carotid plaque progression was significantly increased in subjects with the intermediate and the highest tertiles of the ox-LDL/total LDL particle number ratio (**Table 6**).

In addition, combined group analyses between ox-LDL and blood pressure levels demonstrated in **Supplementary Table 1** showed that the 10-year risk of progression of atherosclerosis was borderline increased in subjects with higher ox-LDL levels and controlled blood

Table 4. Relative risks (RRs) and 95% confidence intervals (CIs) of 10-year risk of progression of carotid atherosclerosis associated with combined groups of oxidized LDL and other LDL-related parameters levels.

Variables	RR (95%CI)	*P*-value
Model 1		
Combined groups of oxidized LDL and LDL cholesterol levels [†]		
Low oxidized LDL and low LDL cholesterol	Reference	
High oxidized LDL and low LDL cholesterol	1.18 (1.00-1.40)	0.050
Low oxidized LDL and high LDL cholesterol	1.28 (1.08-1.51)	0.005
High oxidized LDL and high LDL cholesterol	1.29 (1.13-1.47)	0.002
Model 2		
Combined groups of oxidized LDL levels and total LDL particles number [‡]		
Low oxidized LDL and low total LDL particles number	Reference	
High oxidized LDL and low total LDL particles number	1.19 (1.00-1.41)	0.046
Low oxidized LDL and high total LDL particles number	1.17 (0.98-1.39)	0.093
High oxidized LDL and high total LDL particles number	1.24 (1.09-1.42)	0.001
Model 3		
Combined groups of oxidized LDL levels and small LDL particles number [§]		
Low oxidized LDL and low small LDL particles number	Reference	
High oxidized LDL and low small LDL particles number	1.17 (1.00-1.37)	0.055
Low oxidized LDL and high small LDL particles number	1.00 (0.84-1.20)	0.978
High oxidized LDL and high small LDL particles number	1.19 (1.04-1.37)	0.010

LDL, low density lipoprotein; HDL, high density lipoprotein;
Model 1, Model 2 adjustment for: age, sex, current smoker, type 2 diabetes, SBP, use of anti-hypertension drugs, hs-CRP, TG, HDL-C, and use of statins. Model 3 adjustment for: age, sex, current smoker, type 2 diabetes, SBP, use of anti-hypertension drugs, hs-CRP, and use of statins.
Low oxidized LDL: oxidized LDL < 47.1 U/L; High oxidized LDL: oxidized LDL ≥ 47.1 U/L.
[†] Low LDL cholesterol: LDL cholesterol < 130 mg/dl; High LDL cholesterol: LDL cholesterol ≥ 130 mg/dl;
[‡] Low LDL particle number: LDL particle < 1081.5 nmol/L; High LDL particle number: LDL particle ≥ 1081.5 nmol/L;
[§] Low small LDL particle number: small LDL particle number < 578.5 nmol/L; High small LDL particle number: small LDL particle number ≥ 578.5 nmol/L;

pressure (RR = 1.15, 95% CI 1.00−1.32, $P = 0.053$), whereas the risk was significantly higher in those with higher ox-LDL levels and uncontrolled blood pressure (RR = 1.30, 95% CI 1.12−1.51, $P < 0.001$).

Discussion

In this long-term cohort study in a general population, we found that higher serum ox-LDL levels were associated with an increased 10-year risk of progression of carotid atherosclerosis. This relationship was independent of the cholesterol concentrations, the total number, and the size of LDL particles.

This study extends previous findings from cohort studies showing an association between ox-LDL levels and clinical atherosclerotic events to people with subclinical atherosclerosis. Our previous meta-analysis of prospective cohort studies showed that circulating ox-LDL levels were associated with clinical atherosclerotic cardiovascular events. However, evidence of the relationship between ox-LDL levels and the early stage of atherosclerosis from cohort studies is scarce[4]. Some studies reported that oxidized LDL in circulating immune complexes predicted progression of carotid IMT in type 1 diabetic patients[18, 19], whereas only two cohort studies besides our study have investigated the association between circulating ox-LDL levels and subclinical atherosclerosis in the general population[5, 6]. The Bruneck Study found that baseline levels of oxidized phospholipids present on apolipoprotein B-100 particles (OxPL/apoB) were significantly associated with the 5-year risk of incident atherosclerosis and stenosis in carotid or femoral arteries[6]. Similarly, our analysis demonstrated that baseline circulating ox-LDL levels were also significantly associated with 10-year progression of carotid plaques. Another cohort study that enrolled

Table 5. Relative risks (RRs) and 95% confidence intervals (CIs) of 10-year risk of progression of carotid atherosclerosis associated with baseline oxidized LDL tertiles and combined groups of oxidized LDL and other LDL-related parameters levels in sensitivity analyses.

Variables	RR (95%CI)		
	Subgroup 1 (*N*=776)	Subgroup 2 (*N*=755)	Subgroup 3 (*N*=648)
Oxidized LDL tertiles			
T1	Reference	Reference	Reference
T2	1.16 (1.00-1.34)*	1.16 (1.00-1.33)*	1.16 (1.00-1.36)
T3	1.21 (1.05-1.40)**	1.19 (1.03-1.37)*	1.17 (1.00-1.38)*
Combined groups of oxidized LDL and LDL cholesterol levels			
Low oxidized LDL and low LDL cholesterol	Reference	Reference	Reference
High oxidized LDL and low LDL cholesterol	1.18 (0.99-1.41)	1.21 (1.03-1.43)*	1.24 (1.03-1.48)*
Low oxidized LDL and high LDL cholesterol	1.28 (1.08-1.52)**	1.30 (1.09-1.54)**	1.39 (1.15-1.67)**
High oxidized LDL and high LDL cholesterol	1.29 (1.12-1.48)**	1.26 (1.10-1.45)**	1.28 (1.10-1.49)**
Combined groups of oxidized LDL levels and total LDL particles number			
Low oxidized LDL and low total LDL particles number	Reference	Reference	Reference
High oxidized LDL and low total LDL particles number	1.18 (0.98-1.39)	1.19 (1.00-1.42)	1.24 (1.02-1.51)*
Low oxidized LDL and high total LDL particles number	1.17 (0.98-1.40)	1.16 (0.97-1.38)	1.28 (1.07-1.54)**
High oxidized LDL and high total LDL particles number	1.25 (1.09-1.43)**	1.22 (1.06-1.40)**	1.26 (1.08-1.47)**
Combined groups of oxidized LDL levels and small LDL particles number			
Low oxidized LDL and low small LDL particles number	Reference	Reference	Reference
High oxidized LDL and low small LDL particles number	1.18 (1.00-1.39)*	1.15 (0.98-1.35)	1.14 (0.96-1.36)
Low oxidized LDL and high small LDL particles number	1.01 (0.84-1.21)	0.98 (0.82-1.18)	0.97 (0.80-1.18)
High oxidized LDL and high small LDL particles number	1.19 (1.03-1.36)*	1.17 (1.02-1.35)*	1.15 (0.99-1.35)

* *P*-value < 0.05;
** *P*-value < 0.01;
Subgroup 1: 776 subjects after excluding those having adverse cardiovascular diseases during follow-up; Subgroup 2: 755 subjects after excluding those on statin treatment at baseline; Subgroup 3: 648 subjects after excluding those with any carotid plaque at baseline.
Models adjustment is as specified in Table 3 and 4.

1427 subjects who were free of acute myocardial infarction at baseline failed to show a similar association between ox-LDL levels and subclinical atherosclerosis, possibly because a composite endpoint of carotid IMT and ankle-brachial index was adopted in that study[5].

Moreover, our study showed that the association between ox-LDL levels and subclinical atherosclerosis was independent of LDL-C, total LDL particle number, and LDL particles size. LDL-C has been widely accepted as the cornerstone of atherosclerosis development and progression[20]. Whether the effect of ox-LDL levels on atherosclerosis is specifically caused by oxidative modification of the LDL particle or is merely driven by LDL-C is a key issue that need to be considered when designing any therapeutic approach targeting ox-LDL. The present analysis showed that the association between ox-LDL levels and subclinical atherosclerosis was independent of serum LDL-C concentrations. This finding is in support to the results from the Bruneck Study in which OxPL/apoB recognized by monoclonal antibody E06 was significantly associated with the presence and progression of carotid and femoral atherosclerosis independent of LDL-C[6]. Unlike the Bruneck study, we used monoclonal antibody 4E6, which directly recognizes the modified lysine residues of apolipoprotein B-100 on LDL particles. Oxidized LDL levels measured by both assays have been shown predictive to clinical cardiovascular events in previous meta-analysis[4]. However, it is important to know whether the pro-atherogenic effects of oxidized LDL detected by these assays are independent of the total number of LDL particles. We evaluated the independent effect of ox-LDL using a 2×2 combination between ox-LDL levels and the total LDL particle number. We found that high ox-LDL levels were associated with an increased risk of atherosclerotic progression, regardless of whether the LDL particle number was high or low. Moreover, we found that higher levels of

Table 6. Relative risks (RRs) and 95% confidence intervals (CIs) of 10-year risk of progression of carotid atherosclerosis associated with tertiles of baseline oxidized LDL/LDL particles number ratio.

Variables	RR (95%CI)	*P*-value
Oxidized LDL/LDL particles number ratio tertiles [†]		
T1	Reference	
T2	1.14 (1.00-1.30)	0.049
T3	1.14 (1.00-1.30)	0.058
Age (years)	1.02 (1.01-1.03)	<0.001
Male	0.95 (0.85-1.08)	0.438
Current smoker	1.20 (1.01-1.43)	0.042
Type 2 diabetes	1.09 (0.91-1.30)	0.369
Systolic blood pressure (mmHg)	1.00 (1.00-1.01)	0.030
Use of anti-hypertensive drugs	1.03 (0.92-1.15)	0.655
hs-CRP (mg/L)	1.00 (0.99-1.01)	0.949
Triglyceride (mg/dl)	1.00 (1.00-1.00)	0.956
HDL cholesterol (mg/dl)	0.99 (0.99-1.00)	0.020
Use of statins	0.99 (0.80-1.21)	0.890

LDL, low density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; HDL, high density lipoprotein.
[†]T1 (the lowest tertile): oxidized LDL/LDL particles number ratio <0.041 U/nmol; T2 (the intermediate tertile): 0.041 U/nmol ≤oxidized LDL/LDL particles number ratio <0.049 U/nmol; T3 (the third tertile): oxidized LDL/LDL particles number ratio ≥0.049 U/nmol.

the ox-LDL/total LDL particle number ratio, which represents a higher proportion of oxidation in total LDL particles, was significantly associated with a higher risk of progression of subclinical atherosclerosis. The third LDL-related parameter that needs to be considered is the size of LDL particles because previous studies have shown that small LDL particles are more susceptible to oxidation[21]. Our study showed that the 10-year risk of progression of atherosclerosis was significantly increased in subjects with higher ox-LDL levels independent of the number of small LDL particles. These findings indicate, for the first time, that the relationship between ox-LDL levels and subclinical atherosclerosis is independent of the LDL-related parameters including LDL-C, total LDL particle number, and LDL particles size. The predictive value of ox-LDL on the progression of subclinical atherosclerosis may be mostly driven by oxidative modification itself.

Our findings have important value for enhancing understanding of the atherogenic effects of ox-LDL and developing novel diagnostic and therapeutic approaches to atherosclerosis. Besides uptake through scavenger receptors on macrophages and foam cell formulation in vessel walls, ox-LDL has recently been found to exert other pro-atherogenic effects through LOX-1[22]. LOX-1 is the major ox-LDL receptor on the surface of endothelial cells, resulting in endothelial dysfunction, vessel cell proliferation, and thrombogenesis[2, 23-25]. Our finding that higher circulating ox-LDL levels were associated with subclinical atherosclerosis, independent of other LDL-related parameters, supports the presence of these pro-atherogenic pathways. Our finding also implies that ox-LDL and its mediator molecule could be potential pharmaceutical targets worthy of investigation. Several therapeutic strategies have been developed to reduce ox-LDL levels, such as antioxidants, LOX-1 inhibitors, and antibodies against ox-LDL. Antioxidant supplements or vitamins failed to show the efficacy in prevention of clinical cardiovascular events[26]. One of the potential reasons that may explain for the null result of these trials is that these trials were not performed in subjects who most tend to profit by antioxidant intervention, such as those with high baseline oxidative stress[4, 27]. Additionally, whether antioxidants exert their effects by reducing circulating ox-LDL levels is unclear[28]. Recent studies have shown that the application of LOX-1 antibodies, antisense RNA and miRNA could block LOX-1 and in turn prevent atherogenesis, by inhibiting the binding and internalization of ox-LDL to LOX-1 in human artery endothelial cells and reducing the level of reactive oxygen species[29-32]. More recently, Poulse *et al.* found that treatment with a recombinant human antibody against ox-LDL could reduce the pro-atherogenic macrophage marker in a hypercholesterolemic porcine model with human-like coronary atherosclerosis[33]. These studies have laid a foundation for novel therapeutic approaches to prevent the development or progression of atherosclerosis at an early stage by targeting ox-LDL.

The current study has several limitations. First,

approximately 27% of the initial subjects were not available for a follow-up carotid ultrasound examination. However, the baseline characteristics were not different between the eligible and non-eligible groups. Second, LDL particles are most likely oxidized within the sub-intimal layer, and thus circulating levels of ox-LDL may not accurately reflect LDL retention in atherosclerotic lesions. However, some reports have indicated that higher plasma ox-LDL levels are found in patients with greater deposition of ox-LDL within plaques in the arterial wall[34]. Additionally, circulating ox-LDL may exert a potential atherogenic effect through LOX-1 on the surface of endothelial cells.

In conclusion, the present study indicates that in a population-based cohort, serum ox-LDL levels are associated with 10-year progression of subclinical atherosclerosis. More importantly, this relationship is independent of the cholesterol content, the total number, and the size of LDL particles. These findings indicate that ox-LDL-associated atherogenic effects may largely be derived from oxidative modification of LDL itself, and may have potential diagnostic and therapeutic roles for atherosclerotic cardiovascular disease.

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Conflict of Interest

The authors declare no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

Author Contributions

Shen Gao analyzed data and drafted the manuscript. Jing Liu contributed to initial concepts and study design. All authors contributed to acquisition or interpretation of data. Yue Qi and Shen Gao contributed to laboratory measurements. Jing Liu and Dong Zhao contributed to manuscript revision and supervision. All authors contributed to manuscript final version approval.

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Supplementary Table 1. Relative risks (RRs) and 95% confidence intervals (CIs) of 10-year risk of progression of carotid atherosclerosis associated with combined groups of oxidized LDL and blood pressure levels.

Variables	RR (95%CI)	*P*-value
Combined groups of oxidized LDL and blood pressure levels [†]		
Low oxidized LDL and controlled blood pressure	Reference	
High oxidized LDL and controlled blood pressure	1.15 (1.00-1.32)	0.053
Low oxidized LDL and uncontrolled blood pressure	1.05 (0.88-1.26)	0.569
High oxidized LDL and uncontrolled blood pressure	1.30 (1.12-1.51)	<0.001
Age (years)	1.02 (1.01-1.03)	<0.001
Male	0.96 (0.85-1.08)	0.522
Current smoker	1.19 (1.00-1.42)	0.046
Type 2 diabetes	1.09 (0.91-1.31)	0.337
Use of anti-hypertensive drugs	1.04 (0.93-1.16)	0.533
hs-CRP (mg/L)	1.00 (0.99-1.01)	0.795
Triglyceride (mg/dL)	1.00 (1.00-1.00)	0.678
HDL cholesterol (mg/dL)	0.99 (0.99-1.00)	0.024
Use of statins	0.96 (0.79-1.17)	0.693

LDL, low density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; HDL, high density lipoprotein.
[†] Low oxidized LDL: oxidized LDL <47.1 U/L; High oxidized LDL: oxidized LDL ≥47.1 U/L. Controlled blood pressure: systolic blood pressure <140 mmHg and diastolic blood pressure <90 mmHg; uncontrolled blood pressure: systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg