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A sensitive chemiluminescence imaging immunoassay for simultaneous detection of serum oxidized lipoprotein(a) and low density lipoprotein

Abstract

Background: Oxidized lipoprotein(a) [ox-Lp(a)] and oxidized low density lipoprotein (ox-LDL) levels have been reported to be useful predictors of cardiovascular events. The study developed a chemiluminescence (CL) imaging immunoassay method for simultaneously detecting serum concentrations of ox-Lp(a) and ox-LDL.

Methods: Ox-Lp(a) and ox-LDL levels were measured by CL imaging immunoassay using a disposable immunosensor array as the carrier and a charge-coupled device as the detector, and were studied in 46 acute coronary syndromes (ACS) patients, 58 stable coronary artery disease (CAD) and 61 control subjects.

Results: This method showed good linear relations ($R^2$ > 0.99) in the concentration range of $2.00 \times 10^{-5}$–$2.00 \times 10^{-1}$ and $2.40 \times 10^{-4}$–$2.40 \times 10^{-3}$ U/mL for ox-Lp(a) and ox-LDL, respectively. The detection limits for ox-Lp(a) and ox-LDL were $2.40 \times 10^{-6}$ and $3.00 \times 10^{-5}$ U/mL, respectively. The intra- and inter-assay coefficients of variation (CV) were 4.90%–6.76% and 7.11%–10.06% for ox-Lp(a), and 5.01%–6.04% and 5.47%–9.77% for ox-LDL, respectively. The mean recovery was 99.31% for ox-Lp(a) and 99.57% for ox-LDL, respectively. Significant correlations were observed between ox-Lp(a) levels detected by CL imaging immunoassay and ELISA, and between ox-LDL levels detected by the two methods, respectively. Furthermore, ox-Lp(a) and ox-LDL levels increased in stable CAD, and especially in ACS.

Conclusions: The CL imaging immunoassay provided a simple, sensitive and reliable method for the simultaneous determination of serum ox-Lp(a) and ox-LDL. The clinical monitoring ox-Lp(a) and ox-LDL levels may possess distinctly clinical value for assessment of CAD risk.

Keywords: chemiluminescence imaging; coronary artery disease; immunoassay; lipoprotein(a); low density lipoprotein; oxidation.

Introduction

Dyslipidemia is known to be closely associated with atherosclerosis. Lipoprotein(a) [Lp(a)], as an atherogenic particle, structurally resembles the low density lipoprotein (LDL) but contains a molecule of apolipoprotein(a) [apo(a)] attached to apoB-100 by a disulfide bond [1]. Elevated plasma concentrations of Lp(a) have been considered as an independent risk factor for atherosclerosis [2, 3]. Apo(a) and apoB proteins of Lp(a) can both be oxidatively modified in vivo [4]. Ox-Lp(a) has been reported to play more potent roles than native Lp(a) in atherosclerosis [5]. Several lines of evidence showed that ox-Lp(a) and ox-LDL induced adhesion molecular expression on monocytes, contributing to their recruitment and adhesion to the endothelium, and subsequently promoted intracellular accumulation of cholesteryl esters in macrophages, leading to their transformation into foam cells [6–9]. Circulating ox-LDL, as a useful marker for identifying patients with atherosclerotic disease, has been reported to be closely associated with the high risk of subsequent cardiovascular events [10–13]. Our previous studies have shown that ox-Lp(a) were present in...
newborns and children [14], and increased in rheumatoid arthritis patients with excessive cardiovascular events [15]. Elevated ox-Lp(a) levels have been found to be associated with the presence of acute coronary syndromes (ACS) and stable coronary artery disease (CAD), and with the severity of ACS [16, 17].

Currently, circulating ox-LDL and ox-Lp(a) are most often determined by enzyme-linked immunosorbent assay (ELISA) [4, 18–22]. Ox-LDL levels were also measured by chemiluminescent ELISA using a chemiluminescence (CL) detector [23]. Ox-Lp(a) levels were estimated by the degree of oxidized apo(a) and/or apoB of Lp(a) [4, 21, 22]. However, the above assays cannot simultaneously detect the two oxidized lipoproteins, and their sensitivity is relatively low. Recently, CL immunoassay combines good specificity of immunoreaction with high sensitivity of CL detection and has become a powerful analytical method [24, 25]. This technique has been extensively used for development of multiplex immunoassay [26, 27]. The CL-based imaging assay can provide simple, sensitive and high-throughput method for detection when CL is coupled with a charge-coupled device (CCD) as the detector, which has been successfully applied to microbioassay [28, 29].

Our previous work fabricated a disposable protein chip for multiplex immunoassay of four tumor markers [30]. In the present study, we developed a highly sensitive CL imaging immunoassay for simultaneous detection of serum ox-Lp(a) and ox-LDL by combining a disposable immunosensor array with a cooled low-light CCD, and studied serum ox-Lp(a) and ox-LDL levels in ACS and stable CAD patients.

Materials and methods

Study subjects

The present study included 46 patients with ACS, 58 patients with stable CAD, and 61 control subjects. The control subjects selected from routine health examination were found normal in physical and electrocardiography and laboratory tests, and without diseases such as hyperlipemia, hypertension, diabetes mellitus, or any clinical evidence of atherosclerosis.

The CAD patients in this study were selected from patients under the Department of Cardiology of Jinling Hospital from January 2011 to December 2012, who were undergoing clinically indicated coronary angiography. Angiograms of all the CAD patients showed at least 50% stenosis of one, two, or three coronary arteries. Forty-six patients with ACS included acute myocardial infarction patients and unstable angina with Braunwald classification II or III. Fifty-eight patients with angiographically documented CAD and no cardiac events/procedures for >1 year were considered to have stable CAD. The exclusion criteria of the CAD patients included mild disease of angiography (a stenosis of 10%–50% of the luminal diameter in all the three coronary arteries), prior coronary revascularization and the presence of renal disease. In patients with ACS, blood samples were taken on admission. Blood samples were collected at least 12 h after fasting from control subjects and patients with stable CAD. Serum was separated immediately and stored at −70°C until analysis. All laboratory assays were conducted within 1 year of blood sampling. This study protocol was approved by the Ethics Committee of Jinling Hospital, and all the subjects provided written informed consent.

Isolation and oxidation of Lp(a) and LDL

Lp(a) was purified from plasma of healthy donors by sequential density ultracentrifugation, followed by a gel-filtration chromatography as described [22]. LDL (1.030<d<1.050 g/mL) was obtained from plasma of Lp(a)-negative healthy donors after ultracentrifugation [31]. Purified Lp(a) or LDL was diluted in phosphate-buffered saline (PBS) to a protein concentration of 0.5 mg/mL and incubated with 30 μmol/L CuSO4 for 12 h at 37°C, followed by extensive dialysis, respectively [5]. The degree of oxidation was quantified by relative electrophoretic mobility and the generation of thiobarbituric acid reactive substances.

Sandwich ELISAs

Ox-Lp(a) and ox-LDL were measured by sandwich ELISAs, using rabbit anti-human copper-oxidized LDL (Abcam, Cambridge, UK) as the capture antibody and quantitating with horseradish peroxidase (HRP) labeled monoclonal anti-apo(a) and anti-apoB as previously described, respectively [22, 32]. The capture antibody had high reactivity with ox-LDL, while had almost no cross-reactivity with native LDL [33]. See Supplemental Data 1, which accompanies the article at http://www.degruyter.com/view/j/cclm.2016.52.issue-6/issue-files/cclm.2016.52.issue-6.xml, for additional information.

A pooled fresh-frozen serum sample (from 100 healthy subjects) was used as reference serum of ox-Lp(a) and ox-LDL. The values of ox-Lp(a) and ox-LDL were determined by the ELISAs repeatedly, the protein concentrations of copper-oxidized Lp(a) and LDL were used as the standards, respectively. One unit was defined as equivalent to 1 mg of copper-oxidized Lp(a) or LDL. The values of the reference serum were then assigned to be 0.20 U/mL for ox-Lp(a) and 2.40 U/mL for ox-LDL, respectively.

Preparation of disposable immunosensor array

The details for fabrication of the disposable array have been previously described [27]. See Supplemental Data 2 for more information.

CL imaging immunoassay

The CL imaging immunoassay for simultaneous multianalyte detection was illustrated in Figure 1. The reference serum of ox-Lp(a) and ox-LDL, capture antibody and HRP-labeled antibodies in CL assay
were identical with those of the above ELISAs. Briefly, the 1.5 μL of diluted samples or serial standards were added to the sensing cells on corresponding detection rows, and incubated for 1 h at room temperature. Then 1.5 μL of the diluted HRP-labeled antibodies were added to corresponding sites for 1.5 h, respectively. Finally, 1.5 μL of CL substrate were added to collect the CL signals by CCD. See Supplemental Data 3 for the detailed procedures.

**Statistical analysis**

The values were expressed as mean±SD. Lp(a), ox-Lp(a), ox-LDL and triglyceride (TG) concentrations of non-normal distribution were logarithmically transformed. The differences of variants among groups were analyzed by ANOVA, and the differences between groups were subsequently determined by Fisher LSD test when appropriate. As an approximation of relative risk, the odds ratio (OR) and 95% confidence interval (CI) were calculated for several putative risk factors with univariate and multivariate logistic regression analysis. All study subjects were categorized into the stable CAD, ACS or control group, which was treated as a dependent three-category variable. Correlations between variables were calculated by Pearson’s correlation coefficient. Values of p<0.05 were considered statistically significant.

**Results**

**Base clinical characteristics and lipid concentrations in the study groups**

The baseline clinical characteristics of the patients and control subjects, indications for coronary angiography, lipid concentrations were shown in Table 1. The distributions of Lp(a), and ox-Lp(a) levels in all the studied patients and subjects (n=165) were skewed toward lower values.

**Optimization of assay conditions**

A series of experiments were conducted to establish the optimum reaction conditions for CL imaging immunoassay. The ox-Lp(a) and ox-LDL concentrations used for the optimization experiments were $2.00 \times 10^{-3}$ and $2.40 \times 10^{-2}$ U/mL, respectively. It was found that 0.33 μg/mL capture antibody and 1:100 dilution of HRP-labeled antibodies were enough to maximize CL intensity and minimize non-specific adsorption (Supplemental Data 4, Figure S1). To make the operation convenient, the incubation temperature was performed at room temperature (25°C). The incubation times of 1 and 1.5 h were chosen for the two incubation steps in the sandwich immunoassay (Supplemental Data 4, Figure S2).

The kinetic behavior of the CL reaction catalyzed by HRP-labeled antibodies was studied with a static method. Since CCD imaging needed a long exposure time to collect the weak CL signal and the CL intensity could be retained at the maximum value for 10 min (Figure 2), an exposure time of 10 min was recommended for dynamic integration to collect the CL signal.
Standard curves and detection limits

Under the above optimum conditions, the standard curves obtained by the CL assay for ox-Lp(a) and ox-LDL were shown in Figure 3. The linear range for ox-Lp(a) measurement was 2.00×10⁻⁵–2.00×10⁻¹ U/mL, and that for ox-LDL was 2.40×10⁻⁴–2.40 U/mL. The detection limits for ox-Lp(a) and ox-LDL are 2.40×10⁻⁶ and 3.00×10⁻⁵ U/mL at a signal-to-noise ratio of 3, respectively.

Precision and recovery

With the use of serum samples containing ox-Lp(a) (0.04, 0.41 and 0.79 U/mL) and ox-LDL (0.74, 3.86 and 7.37 U/mL) at low, medium and high levels, the precision of CL imaging immunoassay was evaluated by the intra- and inter-assay coefficients of variation (CV). The intra- and inter-assay CVs were 4.90%–6.76% (n=10) and 7.11%–10.06% (n=5) for ox-Lp(a), and 5.01%–6.04% (n=10) and 5.47%–9.77% (n=5) for ox-LDL, respectively.

To determine the analytical recovery, purified ox-Lp(a) (0.42 and 3.04 U/mL) or ox-LDL (1.53 and 6.01 U/mL) were added to the serum samples containing ox-Lp(a) or ox-LDL of the above three different concentrations, respectively, and then the serum samples were detected with 1:40 dilution. Recoveries of ox-Lp(a) ranged from 92.84% to 102.34% (0.42 U/mL, n=5) and from 98.17% to 102.82% (3.04 U/mL, n=5), respectively. Recoveries of ox-LDL ranged from 94.41% to 101.05% (1.53 U/mL, n=5) and from 91.80% to 108.90% (6.01 U/mL, n=5), respectively.

The immunosensor array could be stored at 4°C. After a storage period of 70 days, the CL responses of ox-Lp(a) and ox-LDL remained 90.67%–95.14% and 91.65%–96.37% of their initial responses, respectively.

Interference study

To investigate the effect of the potentially interfering agents including hemoglobin, total bilirubin, lipid (in terms of TG), heparin and EDTA to the assay, the interfering agents at different concentrations were added to serum sample. It was found that there was no interference from hemoglobin up to 5 mg/mL, total bilirubin up to 200 μmol/L, lipid up to 5.65 mmol/L, heparin up to 0.40 mg/mL, and EDTA up to 2 mg/mL. In addition, to evaluate the effect of high ox-LDL levels on measurement of ox-Lp(a), purified ox-LDL at three different concentrations were added to serum sample, respectively. It was found that
there was no interference from ox-LDL up to 11.36 U/mL. Similarly, the effect of high ox-Lp(a) levels on measurement of ox-LDL was also studied, purified ox-Lp(a) ranged from 1.50 to 5.98 U/mL were added to serum sample, for ox-Lp(a) itself containing oxidized apo(a) and apoB epitopes, thus serum ox-LDL concentrations were correspondingly increased.

**Ox-Lp(a) and ox-LDL levels detected by CL imaging immunoassay and ELISAs**

To assess the analytical reliability and potential application of CL imaging immunoassay, serum samples from 165 subjects were measured by the novel method and the reference ELISAs, respectively. When the levels of analytes were out of the linear ranges, serum samples should be appropriately regulated dilution fold prior to the assay. Based on the concentrations of ox-Lp(a) or ox-LDL detected by ELISA in all the studied patients and subjects, there were significant correlations between ox-Lp(a) levels detected by the two methods ($R^2=0.739$, $p<0.001$; $Y=0.44X+0.13$, SD=0.14, n=165), and between ox-LDL levels detected by the two assays ($R^2=0.824$, $p<0.001$; $Y=0.57X+0.95$, SD=1.04, n=165), respectively, where $Y$ represented ox-Lp(a)/ox-LDL levels detected by ELISA and $X$ represented those detected by CL assay (Figure 4). Furthermore, the 75th percentile value of ox-Lp(a) or ox-LDL measured by ELISA in the controls was chosen as the cut-off point, respectively. Cut-off values for ox-Lp(a) and ox-LDL were 0.24 U/mL and 2.67 U/mL, respectively. According to the cut-off value of ox-Lp(a) or ox-LDL, the total sample was divided into two subgroups, low-level group and high-level group. The correlations were observed between ox-Lp(a) levels ($r=0.925$, $p<0.001$) or ox-LDL ($r=0.905$, $p<0.001$) measured by two assays in the low-level group, and between ox-Lp(a)
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(r = 0.654, p < 0.001) or ox-LDL levels (r = 0.728, p < 0.001) in the high-level group, respectively.

Association with presence of ACS and stable CAD

Compared to the controls, ox-Lp(a) and ox-LDL levels detected by the two assays were found increased in both ACS and stable CAD patients. Furthermore, ox-Lp(a) and ox-LDL concentrations in the ACS patients were significantly higher than those in the stable CAD patients (Table 2).

The univariate and multivariate logistic regression analyses were next performed to evaluate the relationship of the two oxidized lipoproteins with stable CAD and ACS, respectively. As shown in Table 3, the univariate analysis revealed that the presence of ox-Lp(a) detected by CL assay or ELISA was a risk factor for stable CAD, and especially for ACS, respectively. Similarly, the presence of ox-LDL was also a risk factor for both stable CAD and ACS.

In multivariate analysis, adjusting for age, sex and serum lipid levels, the presence of ox-Lp(a)/ox-LDL detected by two assays was still revealed to be a risk factor for both stable CAD and ACS (Table 3).

Discussion

In this study, we developed and optimized a novel CL imaging immunoassay for simultaneously measuring serum ox-Lp(a) and ox-LDL levels. Significant correlations were observed between ox-Lp(a) levels detected by the new method and ELISA, and between ox-LDL levels by the two assays, respectively. In addition, our study also demonstrated that serum ox-Lp(a) and ox-LDL levels increased in stable CAD, and especially in ACS.

Ox-Lp(a) and ox-LDL levels were determined by CL assay, using rabbit anti-human oxidized LDL as the capture antibody and quantitating with monoclonal anti-apo(a) and anti-apoB enzyme conjugate, respectively. The concentration of capture antibody was sufficient to measure the levels of ox-Lp(a) and ox-LDL in diluted serum, and the monoclonal antibody against apo(a) reacted with Lp(a), but not with plasminogen or LDL [22]. Thus, the CL assay for ox-Lp(a) had specificity for the ox-Lp(a), while had no reactivity with oxidized LDL and plasminogen. In addition, our study also confirmed that high levels of ox-LDL had no effect on measurement of ox-Lp(a). The antibodies against oxidized apoB can both combine with oxidized apoB epitopes of LDL and Lp(a), so this assay for ox-LDL could quantitate oxidized LDL epitopes in both LDL and Lp(a). In fact, all the current assay methods related to LDL index, such as LDL cholesterol, apoB and ox-LDL, include those of Lp(a), and it is necessary and conventional to

![Figure 4](image-url)  
Figure 4 Correlation between ox-Lp(a) levels detected by CL assay and ELISA in total subjects (n = 165) (A). Correlation between ox-LDL levels detected by CL assay and ELISA in total subjects (n = 165) (B).

| Table 2 Ox-Lp(a), ox-LDL concentrations in ACS, stable CAD and control groups. |
|-----------------|--------|----------------|-----------------|-----------------|
| Variable        | Method | ACS  (n=46)    | Stable CAD (n=58) | Control (n=61)  |
| Ox-Lp(a), U/mL  | CL     | 0.49±0.50bd   | 0.26±0.30*       | 0.17±0.18       |
| Ox-Lp(a), U/mL  | ELISA  | 0.37±0.24bcd  | 0.26±0.22a       | 0.17±0.13       |
| Ox-LDL, U/mL    | CL     | 4.67±2.73bcd  | 3.10±1.96a       | 1.55±1.00       |
| Ox-LDL, U/mL    | ELISA  | 3.55±1.80bcd  | 2.77±1.61a       | 1.80±1.14       |

Data are presented as the mean value±SD. CL represents CL imaging immunoassay. Compared with control, *p<0.05; **p<0.01; compared with stable CAD, *p<0.05; **p<0.01.
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accurately estimate LDL level in clinic. The present ELISA and CL assay for ox-LDL also detected the oxidized apoB epitopes in Lp(a).

The fabrication of immunosensor array was simple and low cost, and the array could be stored at 4°C for 70 days without obvious signal change, indicating that it had acceptable stability. The high sample throughput depended on the number of sensing cells per array, and the CCD could simultaneously detected three arrays. Collecting for only 10 min, the detection throughput could be greatly increased when more arrays were used for parallel incubation and immunoassay. Due to the high analytical sensitivity, the CL assay required only a small amount of samples. The detection ranges of ox-Lp(a)/ox-LDL by this method were wide. Besides, the acceptable precision and accuracy of the assay were suitable to detect serum ox-Lp(a) and ox-LDL.

A significant correlation was found between ox-Lp(a)/ox-LDL levels detected by the two assays. Furthermore, the correlation between ox-Lp(a)/ox-LDL levels measured by the two assays in the low-level group was stronger than that in the high-level group, respectively. The increase value of CL intensity or ELISA absorbance to oxidized lipoproteins levels ratio was used to evaluate the analytical ranges. The ratios of CL assay were greater than those of ELISAs at high concentrations [ox-Lp(a), 0.17 a.u./U/mL vs. 0.09 AU/U/mL; ox-LDL, 0.18 a.u./U/mL vs. 0.12 AU/U/mL, respectively], while they were similar at low concentrations [ox-Lp(a), 0.17 a.u./U/mL vs. 0.18 AU/U/mL; ox-LDL, 0.18 a.u./U/mL vs. 0.20 AU/U/mL, respectively]. These findings indicated that the detection ranges of CL assay were wider than that of ELISAs, especially in high-level group, which caused different correlation coefficient between the high- and low-level groups. The clinical data also showed that both ox-Lp(a) and ox-LDL levels measured by CL assay in the ACS patients exhibited more significant increase than those by ELISAs compared with controls.

Ox-Lp(a) and ox-LDL concentrations were found increased in both the ACS and stable CAD patients. Interestingly, ox-Lp(a) and ox-LDL levels were significantly higher in the ACS patients than those in the stable CAD patients, which is similar to what we previously reported [16, 17, 22, 31, 34], which may be partly caused by the release of oxidized lipoproteins derived from ruptured or permeable plaque into the circulation. Furthermore, logistic regression analysis revealed that the presence of ox-Lp(a)/ox-LDL was a risk factor for stable CAD, and especially for ACS. Therefore, serum ox-Lp(a) and ox-LDL levels measured by CL assay as well as ELISAs may be useful risk biomarkers for stable CAD, and especially for ACS. The diagnostic implications of the CL assay remain to be further established in large sample studies.

The main limitation of this study was the relatively low reproducibility of CL assay, which may be caused by the lag time of CL reaction initiation when CL substrate was manually added to the array. The limitation of reproducibility may be avoided by automated pipetting systems and the application of the selection of CL enhancer and the signal amplification technology including gold nanoparticles, carbon nanotubes or silica nanoparticles which could be used as carriers to bind a high loading of HRP or artificial enzyme mimics, which are also our future research directions.

In conclusion, we developed a sensitive CL imaging method for simultaneously detecting serum levels of

### Table 3 Univariate and multivariate logistic regression analysis of risk factors for stable CAD and ACS.

<table>
<thead>
<tr>
<th>Group</th>
<th>Method</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted OR (95% CI)</td>
<td>p-Value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Stable CAD</td>
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<td></td>
</tr>
<tr>
<td>Ox-Lp(a)</td>
<td>CL</td>
<td>6.00 (1.04, 34.68)</td>
<td>0.045</td>
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<td>Ox-Lp(a)</td>
<td>ELISA</td>
<td>29.70 (22.89, 3065.06)</td>
<td>0.005</td>
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<tr>
<td>Ox-LDL</td>
<td>CL</td>
<td>2.29 (1.62, 3.22)</td>
<td>&lt;0.001</td>
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<tr>
<td>Ox-LDL</td>
<td>ELISA</td>
<td>1.77 (1.30, 2.42)</td>
<td>&lt;0.001</td>
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<tr>
<td>ACS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ox-Lp(a)</td>
<td>CL</td>
<td>26.51 (5.00, 152.89)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ox-Lp(a)</td>
<td>ELISA</td>
<td>264.86 (2.77, 318.15)</td>
<td>&lt;0.001</td>
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<tr>
<td>Ox-LDL</td>
<td>CL</td>
<td>3.08 (2.14, 4.45)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>ELISA</td>
<td>2.31 (1.66, 3.21)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

In univariate and multivariate logistic regression analyses, stable CAD, ACS or the control group was treated as a dependent three-category variable. OR was considered significant when the lower limit of the 95% CI was >1.0. p<0.05 was considered significant. *Reference category: control. bOnly one variable, ox-Lp(a) or ox-LDL was included in the model. *The age, sex and serum lipid levels were adjusted in the model. CL represents CL imaging immunoassay.
ox-Lp(a) and ox-LDL. The presence of ox-Lp(a) as well as ox-LDL was an important risk factor for both stable CAD and ACS. The CL assay of ox-Lp(a) and ox-LDL may provide a new approach to investigate the causal roles of ox-Lp(a) and ox-LDL in atherosclerotic cardiovascular disease in a prospective study and to explore the exact pathogenic roles of ox-Lp(a) and ox-LDL.

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Conflict of interest statement

Authors’ conflict of interest disclosure: The authors have no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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