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ORIGINAL ARTICLE

Electronegative low-density lipoprotein of patients with metabolic syndrome induces pathogenesis of aorta through disruption of the stimulated by retinoic acid 6 cascade

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Keywords
Aorta, Electronegative low-density lipoprotein, Stimulated by retinoic acid 6

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ABSTRACT

Aims/Introduction: Electronegative low-density lipoprotein (L5) is the most atherogenic fraction of low-density lipoprotein and is elevated in people with metabolic syndrome (MetS), whereas the retinol-binding protein 4 receptor (stimulated by retinoic acid 6 [STRA6]) cascade is disrupted in various organs of patients with obesity-related diseases. Our objective was to investigate whether L5 from MetS patients capably induces pathogenesis of aorta through disrupting the STRA6 cascade.

Material and Methods: We examined the *in vivo* and *in vitro* effects of L5 on the STRA6 cascade and aortic atherogenic markers. To investigate the role of this cascade on atherosclerotic formation, *crbp*1 transfection was carried out *in vitro*.

Results: This study shows that L5 activates atherogenic markers (p38 mitogen-activated protein kinases, pSmad2 and matrix metallopeptidase 9) and simultaneously suppresses STRA6 signals (STRA6, cellular retinol-binding protein 1, lecithin-retinol acyltransferase, retinoic acid receptor-α and retinoid X receptor-α) in aortas of L5-injected mice and L5-treated human aortic endothelial cell lines and human aortic smooth muscle cell lines. These L5-induced changes of the STRA6 cascade and atherogenic markers were reversed in aortas of LOX1$^{-/-}$ mice and in LOX1 ribonucleic acid-silenced human aortic endothelial cell lines and human aortic smooth muscle cell lines. Furthermore, *crbp1* gene transfection reversed the disruption of the STRA6 cascade, the phosphorylation of p38 mitogen-activated protein kinases and Smad2, and the elevation of matrix metallopeptidase 9 in L5-treated human aortic endothelial cell lines.

Conclusions: This study shows that L5 from MetS patients induces atherogenic markers by disrupting STRA6 signaling. Suppression of STRA6 might be one novel pathogenesis of aorta in patients with MetS.

INTRODUCTION

Many studies have reported that cardiac and vascular damage is developed in people with metabolic syndrome (MetS), such as left ventricular diastolic dysfunction, left ventricular hypertrophy and plaque formation of carotid arteries[1–4]. MetS is an entity characterized with multiple risk factors for cardiovascular disease, including abdominal obesity, high-normal or high blood glucose, triglyceride, blood pressure levels and low high-density lipoprotein cholesterol level[5,6]. Recently, we have observed that human electronegative low-density lipoprotein (LDL; L5) is elevated in people with MetS[7], as well as patients

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with hypercholesterolemia[8], type 2 diabetes[9] and acute myocardial infarction[10]. L5 can tremendously accelerate atherosclerotic processes, such as endothelial cell apoptosis[11,12], platelet aggregation[10], C-reactive protein overproduction[13] and impairment of endothelial cell regeneration[9]. Other investigators have also reported that electronegative LDL can promote triglyceride accumulation in cardiomyocytes[14] and induce inflammasome activation in human macrophages[15]. Recently, we reported that L5 isolated from MetS patients could induce adipose inflammation by promoting macrophage maturation and infiltration into adipose tissue[7]. A recent study showed that chemical composition and atherogenic effects of the LDL subfractions in healthy controls and MetS patients were different. The significant differences in MetS patients and in healthy controls were protein concentration, triglyceride, and cholesteryl esters[16]. Therefore, we supposed that MetS-L5 had more cytotoxicity in atherogenesis. In circulation, retinol is carried by retinol-binding protein 4 (RBP4) that connects with transthyretin (TTR)[17,18]. The retinol–RBP4–transthyretin complex is recognized by the special receptor, termed "stimulated by retinoic acid 6" (STRA6), which transports retinol into cells from the retinol–RBP4–transthyretin complex by the participation of STRA6[19–21] and retinol-metabolizing enzymes, including cellular retinol-binding protein 1 (CRBP1), and lecithin-retinol acyltransferase (LRAT)[22,23]. The intracellular retinol is thereafter metabolized into retinoic acid (RA), which exerts its biological function through retinoic acid receptors (RARs) and retinoid X receptor (RXR)[17,18]. It has been shown that disruption of retinol-signaling pathways by dietary vitamin A deprivation or genetic inhibition of retinol signals can adversely modulate hepatic lipid metabolism[24,25], adipogenesis[26,27], obesity[28] and glucose metabolism[29]. Most importantly, Trasino *et al.* showed for the first time that high-fat diet-fed, *ob*/*ob* and *db*/*db* mice had greatly reduced retinol, CRBP1 and RAR (RARα, RARβ2 and RARγ) levels in the liver, pancreas, lungs and kidneys[29]. They also showed that increasing severity of fatty liver disease in humans correlates with reductions in hepatic retinol, retinol transcriptional signaling and *crbp1* levels in hepatic stellate cells[29].

Several studies have shown that exogenous administration of RA and $RAR\beta_2$ agonist can inhibit atherosclerosis in animal experiments[30–33], despite conflicting results of clinical trials[31,34]. Retinol signals are expressed in blood vessel cells and the aorta[35]; however, whether these atherosclerotic risk factors, such as dyslipidemia, alter STRA6 signaling pathway in arteries has not been elucidated. Here, we explored whether L5 isolated from people with MetS could alter STRA6, CRBP1, LRAT, RARα and RXRα, and whether the disruption of the STRA6 cascade is associated with L5-induced atherosclerotic formation.

METHODS

Materials

The primary antibodies against LOX1, CRBP1, RARα, RARγ, RXRα, LRAT, anti-pSmad2, anti-Smad2, transforming grown factor-β_1 ($TGF\beta_1$), caspase 3 and matrix metallopeptidase 9 (MMP9) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against STRA6, p38 mitogen-activated protein kinases (p38MAPK) and anti-p-p38MAPK antibody were purchased from ABGENT (San Diego, CA, USA). Anti-actin antibody was purchased from Millipore (Temecula, CA, USA). Horseradish peroxidase-conjugate antibody was purchased from Millipore (Temecula).

L5 isolation

In the present study, written informed consent was obtained from each participant who was diagnosed with Mets. All procedures were carried out according to the Declaration of Helsinki, and approved by the institutional review board of Kaohsiung Medical University Hospital (KMUH-IRB-20130397). Research records and informed consent of participants were conserved in KMUH-IRB. Human L5 was isolated from Mets patients (n = 29) who had three or more of the following criteria: (i) waist circumference >90 cm in men and >80 cm in women; (ii) triglyceride >150 mg/dL; (iii) high-density lipoprotein cholesterol <40 mg/dL in men and <50 mg/dL in women; (iv) blood pressure >130/85 mmHg or taking antihypertensive medication; and (v) fasting plasma glucose >100 mg/dL and/or taking antidiabetic agents. The standard operating procedures of biochemical analysis were carried out at the medical laboratory of Kaohsiung Medical University Hospital, Kaohsiung, Taiwan. All participants were expected to fast for at least 8 h before the sampling time and were in a stable condition in that they did not have acute illness. No participants were receiving any operation within 3 months and none were pregnant. Venus blood samples (30 mL) from MetS patients were freshly collected in blood tubes supplemented with 5 mmol/L ethylenediaminetetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA), protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA) and 1% penicillin/streptomycin (Sigma-Aldrich). LDL (density 1.019–1.063 g/cm^3) was isolated from whole blood samples of Mets patients (20 mL) by the ultracentrifugation of sequential potassium bromide density and was then treated by 5 mmol/L ethylenediaminetetraacetic acid for preventing LDL samples from oxidation. Isolated LDL was injected into Uno-Q12 anion-exchange column (Bio-Rad Laboratories, Berkeley, CA, USA) of an ÄKTA fast protein liquid chromatography system (GE Healthcare Life Sciences, Pittsburgh, PA, USA) to be separated into L1 and L5 subfractions. L1 and L5 subtractions were then eluted by multistep linear gradient buffer (1 mol/L NaCl, 0.02 mol/L Tris-HCl, 0.5 mmol/L ethylenediaminetetraacetic acid, pH 8.0) at the flow rate of 2 mL/min, and the absorbance was detected at 280 mm. The effluents of L1 and L5 subtractions were individually concentrated with centriprep centrifugal filters (YM-30; Merck Millipore, Danvers, MA, USA) and passed through 0.22-μm filters (Merck Millipore) under N_2-sealed condition at 4°C.

Animal studies

Male C57B/6J mice were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Male LOX1 knockout mice ($LOX1^{-/-}$) were donated by Professor Tatsuya Sawamura (Shinshu University, Matsumoto, Japan). The genome of $LOX1^{-/-}$ mice was characterized by: (i) the 6–8th exon of *LOX1* gene is replaced with a neomycin-resistant gene in the homologous *LOX1* gene; and (ii) the thymidine kinase gene is replaced downstream of the *LOX1* gene fragment for negative selection[36]. All mice were fed with chow diet, and lived under a 12-h light–dark cycle and pathogen-free facility. Eight-week-old mice ($n = 3$) were injected with 150 μL saline, L1 (1 mg/kg) or L5 (1 mg/kg) through the tail vein every day for 4 weeks. At the end of the experiment, mice were anesthetized with chloral hydrate for isolation of aortas. This study protocol was approved by The Institutional Animal Care and Use Committee of Kaohsiung Medical University for all animal experiments (IACUC No. 102149).

Cell culture

Human aortic endothelial cell line (HAEC; ATCC® PCS-100-011™) and aortic smooth muscle cell line (HASMC; ATCC® PCS-100-012™) were incubated in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc.), 100 U/mL penicillin (Thermo Fisher Scientific Inc.) and 0.1 mg/mL streptomycin (Thermo Fisher Scientific Inc.) at 37°C under a humidified atmosphere containing 5% CO_2. For starvation, HAECs and HASMCs were incubated with serum-free DMEM (Thermo Fisher Scientific Inc.) for 24 h. After starvation, HAECs and HASMCs were treated with native human L1 (50 μg/mL) or L5 (50 μg/mL) and inculcated in serum-free DMEM (Thermo Fisher Scientific Inc.) for 24 h.

Small interfering ribonucleic acid transfection

Human LOX1 small interfering ribonucleic acid (siRNA) and negative control siRNA were obtained from Santa Cruz Biotechnology Inc. For LOX1 silencing, HAEC and HASMC cells were seeded in six-well plates at a density of 2×10^5 cells/well in 2 mL antibiotic-free DMEM at 37°C under a humidified atmosphere containing 5% CO_2. Until HAEC and HASMCs were grown, covering 80% of the area per well, HAECs and HASMCs were incubated in a mixture of negative control scramble siRNA or LOX1 siRNA, and transfection reagent (Santa Cruz Biotechnology Inc.) for 7 h. After siRNA transfection, HAECs and HASMCs were placed in serum-free DMEM for 24-h starvation, and then stimulated with PBS, native L1 (50 μg/mL) or L5 (50 μg/mL) for 24 h.

CRBP1 complementary deoxyribonucleic acid transfection

Our previous study showed that the *crbp1* gene transfection could reverse L5-disrupted STRA6 signaling in renal tubular cells[37]. Therefore, *crbp1* gene transfection was carried out to investigate whether reverse decline of STRA6 signaling under L5-stimulation in aortic cells occurred. The plasmid DNA of cytomegalovirus 6-green fluorescent protein (pCMV6-GFP) vector and human *crbp1* complementary deoxyribonucleic acid (gene number NM-002899) were purchased from OriGene Technologies Inc. (Rockville, MD, USA). The *crbp1* complementary deoxyribonucleic acid was combined with the pCMV6-GFP vector (OriGene Technologies, Inc.) at the Sgfl/Mlul site by following the manufacturer's instructions. The pCMV6-*crbp1*-I-GFP or pCMV6-GFP vector was packaged by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and transfected into HAECs. The pCMV6-*crbp1*-I-GFP or pCMV6-GFP vector-transfected HAECs were incubated in Opti-MEM (Invitrogen) at 37°C for 5 h, and then placed in non-antibiotic and serum-free DMEM (Invitrogen). For the experiment, the pCMV6-*crbp1*-I-GFP or pCMV6-GFP vector-transfected HAECs were treated with phosphate-buffered saline, native L1 (50 μg/mL) or L5 (50 μg/mL) for 24 h.

Western blot

Protein samples of aorta, HAECs and HASMCs were extracted with M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA), processed with electrophoresis in sodium dodecyl sulphate polyacrylamide gel electrophoresis, then total proteins of sodium dodecyl sulphate polyacrylamide gel electrophoresis were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore) with electrophoresis. For blocking non-specific antigen, the PVDF membrane was immersed in Tris-buffered saline 0.2% Tween 20 (TBS-T) and 5% skim milk at 4°C overnight. Then, the PVDF membrane was immersed in TBS-T containing 5% skim milk and primary antibodies for detecting L5 receptor (LOX1), STRA6 signaling (STRA6, CRBP1, RARα and RXRα), phosphorylation of atherogenic p38MAPK (p-p38MAPK and p38MAPK)[38,39] and Smads pathway (pSmad2 and Smad2)[40], and markers of atherogenesis ($TGF\beta_1$, caspase 3 and MMP9)[38–41] in aortic cells. After washing with TBS-T, the PVDF membrane was immersed in TBS-T containing 5% skim milk and a 1:10,000 dilution of horseradish peroxidase-conjugated antibody. The chemiluminescence signal on PVDF membrane was induced by an ECL detection kit (Millipore) and was captured by luminescence imaging system. The densities of western blots were calculated by a luminescence imaging system.

Statistical analysis

Statistical analysis used the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Data were evaluated by mean ± standard error, whereas one-way ANOVA and the Bonferroni test were used to measure significant differences between experimental and control groups. Probability values of $P < 0.05$ were considered as significant difference.

RESULTS

Biochemical characteristics of patients with MetS

The study group included 19 men and 10 women. Their mean values of age, waist circumference, andsystolic and diastolic blood pressure were 57.2 ± 10.3 years, 92.4 ± 12.1 cm,

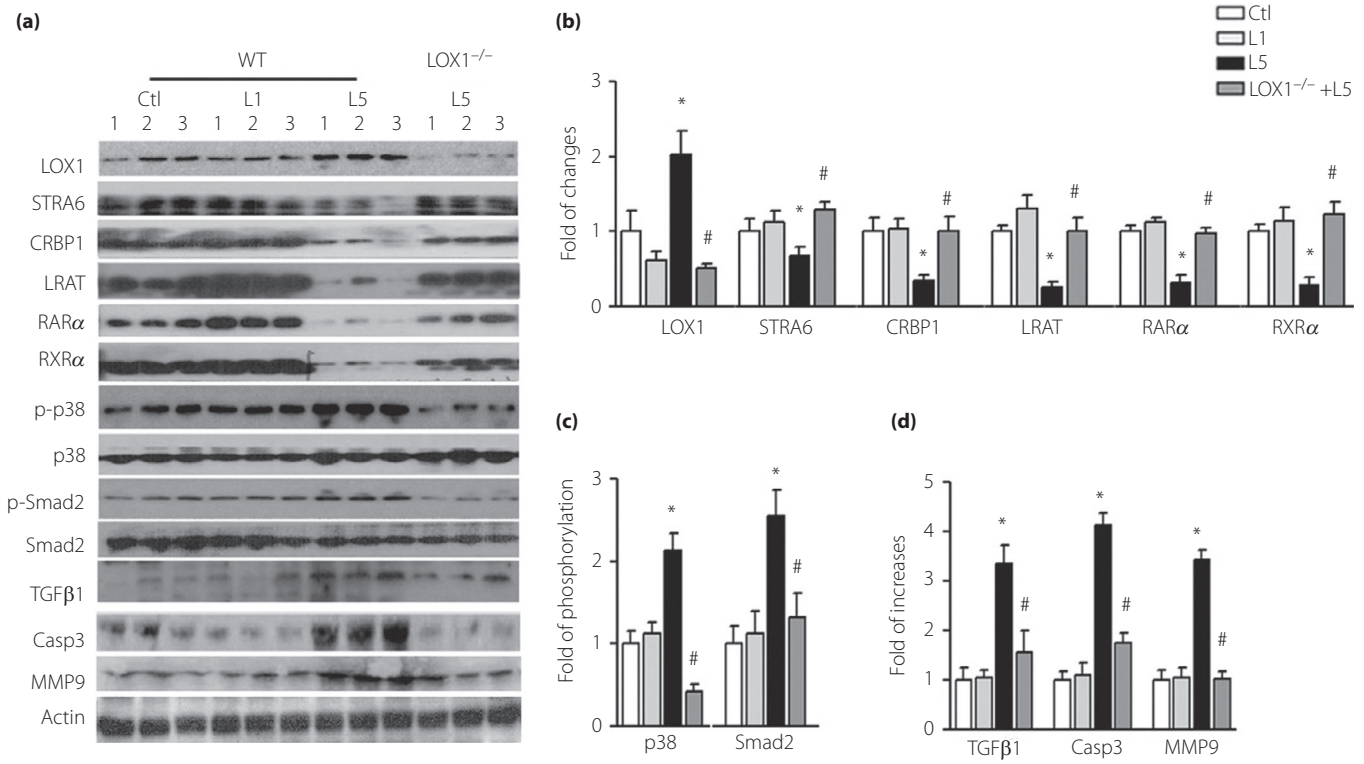

Figure 1 | Stimulated by retinoic acid 6 (STRA6) cascades decrease and markers of atherosclerosis increase in the aortas of electronegative low-density lipoprotein (L5)-injected mice. The protein of aorta samples (n = 3) was extracted from saline-injected (control [Ctl]), L1-injected (L1) and L5-injected (L5) mice, as well as L5-injected LOX1$^{-/-}$ mice (LOX1$^{-/-}$+L5) after injection for 4 weeks. (a) Western blots showed that L5 decreased aortic STRA6, cellular retinol-binding protein 1 (CRBP1), lecithin-retinol acyltransferase (LRAT), retinoic acid receptor (RAR)α and retinoid X receptor (RXR)α, but increased aortic LOX1, p-p38 mitogen-activated protein kinases (p-p38), pSmad2, transforming grown factor-β_1 (TGFβ_1), caspase 3 (Casp3) and matrix metallopeptidase 9 (MMP9) in L5-injected mice. In LOX1$^{-/-}$ mice, these changes caused by L5 were ameliorated. (b) Bar graphs showed that LOX1 increased, and STRA6, CRBP1, LRAT, RARα and RXRα decreased in L5-injected mice. These changes caused by L5 were recovered in L5-injected LOX1$^{-/-}$ mice. (c) Bar graphs show that the p-p38/p38 and pSmad2/Smad2 ratio increased in L5-injected mice. These changes were reversed in L5-injected LOX1$^{-/-}$ mice. (d) TGFβ_1, caspase 3 and MMP9 levels of L5-injected mice increased. These changes were also recovered in L5-injected LOX1$^{-/-}$ mice. All results are presented as the mean ± standard error. *$P < 0.05$ versus Ctl and L1; #$P < 0.05$ versus L5. WT, wild-type.

142.0 ± 23.1 mmHg and 87.0 ± 15.4 mmHg, respectively, whereas mean glucose, triglyceride, high-density lipoprotein cholesterol, LDL-cholesterol values and L5 were 136.2 ± 49.6 mg/dL, 259.6 ± 209.1 mg/dL, 45.4 ± 9.7 mg/dL, 142.2 ± 41.8 mg/dL and 7.3 ± 9.8 mg/dL, respectively. LDL from patients was distinctly divided into five subfractions (L1–L5) according to electronegativity by using fast protein liquid chromatography with an anion-exchange column. The fast protein liquid chromatography analysis showed that the proportion of L5 levels was 5.3 ± 6.9% in MetS patients. The value was significantly higher than that (2.1 ± 1.4%) in healthy participants in our previous study[7].

STRA6 signals decrease, but LOX1 and atherosclerotic biomarkers increase in the aortas of L5-injected mice

LOX1 expression significantly increased, but STRA6, CRBP1, LRAT, RARα and RXRα expression decreased in the aortas of L5-injected C57B6/J mice in comparison with saline- and L1-injected mice (Figure 1a,b). Furthermore, p-p38 and pSmad2 (Figure 1a,c), TGFβ_1, caspase 3 and MMP9 (Figure 1a, d) expressions increased in the aortas of L5-injected mice as compared with saline- and L1-treated groups.

L5-induced changes of the STRA6 cascade and atherosclerotic biomarkers are reversed in the aorta of L5-injected LOX1$^{-/-}$ mice

All these changes of STRA6, CRBP1, LRAT, RARα, RXRα, p-p38/p38, pSmad/Smad2, TGFβ_1, caspase 3 and MMP9 protein levels in the aortas of L5-injected mice were reversed in L5-injected LOX1$^{-/-}$ mice (Figure 1a-d).

L5 reduces the STRA6 cascade, but increases LOX1 and atherosclerotic biomarkers in human aortic endothelial cells

To further verify these results observed *in vivo*, we added native L1 and L5 to HAECs with control siRNA- and LOX1

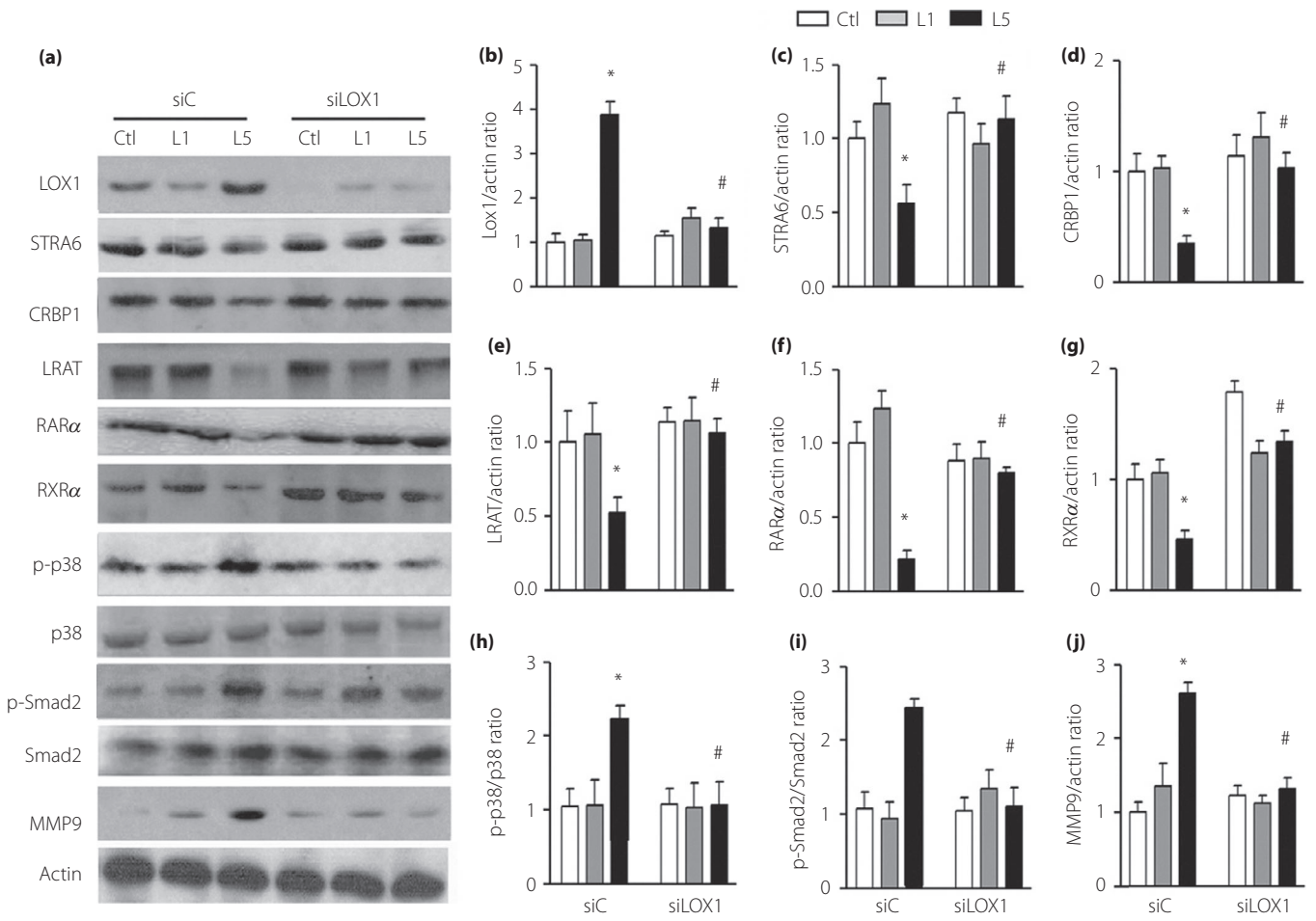

Figure 2 | Electronegative low-density lipoprotein (L5) suppresses stimulated by retinoic acid 6 (STRA6) cascades and induces markers of atherosclerosis in human aortic endothelial cell lines (HAECs). (a) Western blots showed the expression of LOX1, STRA6, cellular retinol-binding protein 1 (CRBP1), lecithin-retinol acyltransferase (LRAT), retinoic acid receptor (RAR)α, retinoid X receptor (RXR)α, p-p38 mitogen-activated protein kinases (p-p38), pSmad2, and matrix metallopeptidase 9 (MMP9) in control small interfering ribonucleic acid (siC)-transfected and LOX1 small interfering ribonucleic acid (siLOX1)-transfected HAECs ($n = 3$) after phosphate-buffered saline (control [Ctl]), L1 or L5 treatment for 24 h. In L5-treated HAECs, the quantitative analysis showed that (b) LOX1 protein level significantly increased; but (c) protein levels of STRA6, (d) CRBP1, (e) LRAT, (f) RARα and (g) RXRα decreased; (h) p-p38/p38 and (i) pSmad2/Smad2 ratios increased; and (j) protein level of MMP9 increased. These changes were reversed in LOX1 small interfering ribonucleic acid-transfected HAECs under L5 treatment. All results are presented as the mean ± standard error. $^{*}P < 0.05$ versus Ctl- and L1-treated siC group; $^{\#}P < 0.05$ versus L5-treated siC group.

siRNA-transfected HAECs for 24 h. L5 treatment strongly increased LOX1 (Figure 2a,b), but decreased STRA6, CRBP1, LRAT, RARα and RXRα levels (Figure 2a,c–g), and increased p-p38, pSmad2 and MMP9 in aortic endothelial cells (Figures 2a,h–j) in control siRNA-transfected HAECs. These changes caused by L5 treatment were reversed by LOX1 siRNA transfection (Figure 2a,b–j) in LOX1 siRNA-transfected HAECs.

L5 reduces the STRA6 cascade, but increases LOX1 and atherosclerotic biomarkers of in human aortic smooth muscle cells

In HASMCs with control siRNA transfection, L5 treatment significantly increased LOX1 (Figure 3a,b), but decreased STRA6, CRBP1, LRAT, RARα and RXRα levels (Figure 3a,c–g), and increased p-p38, pSmad2 and MMP9 (Figure 3a,h–j) as compared with phosphate-buffered saline and L1 treatment. These changes caused by L5 treatment were reversed by LOX1 siRNA transfection (Figure 3a,b–j) in LOX1 siRNA-transfected HASMCs.

CRBP1 transfection reverses the decrease of STRA6 cascades and the increase of atherosclerotic biomarkers in L5-treated aortic endothelial cells

STRA6 cascade-mediated retinol transport requires the presence of CRBP1. We investigated whether *crbp1* transfection could repair STRA6 cascades and this rescue could inhibit L5-induced

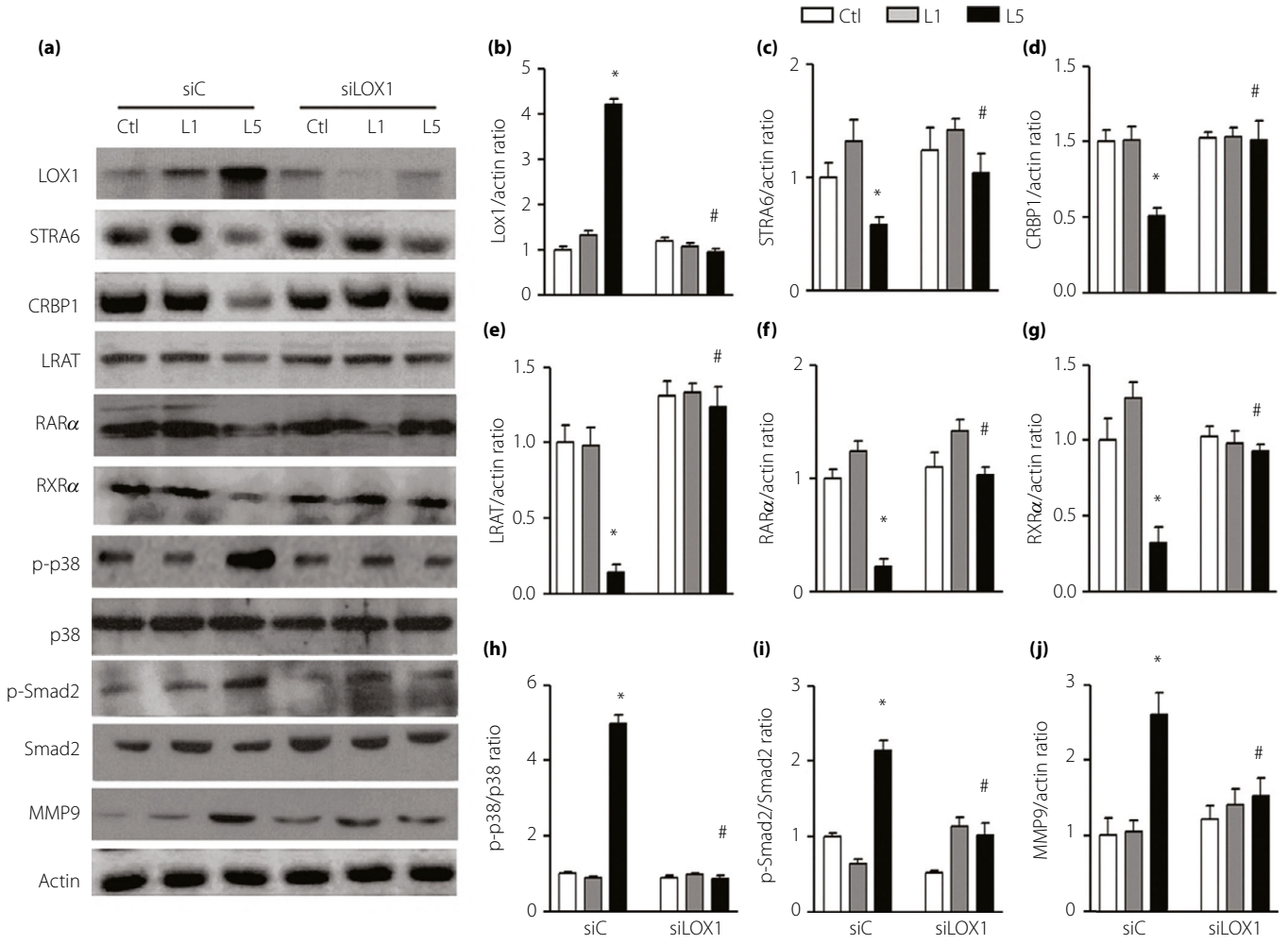

Figure 3 | Electronegative low-density lipoprotein (L5) suppresses stimulated by retinoic acid 6 (STRA6) cascades and enhances markers of atherosclerosis in human aortic smooth muscle cell lines (HASMCs). (a) Western blots showed LOX1, STRA6, cellular retinol-binding protein 1 (CRBP1), lecithin-retinol acyltransferase (LRAT), retinoic acid receptor (RAR)α, retinoid X receptor (RXR)α, p-p38 mitogen-activated protein kinases (p-p38), p-Smad2 and matrix metallopeptidase 9 (MMP9) protein expression in control small interfering ribonucleic acid (siC)-transfected and LOX1 small interfering ribonucleic acid (siLOX1)-transfected HASMCs (n = 3) after phosphate-buffered saline (control [Ctl]), L1 or L5 treatment for 24 h. Quantitative analysis showed that L5 treatment (b) increased LOX1, (c) decreased STRA6, (d) CRBP1, (e) LRAT, (f) RARα and (g) RXRα protein levels, but (h) increased p-38/p38 and (i) pSmad2/Smad2 ratios, and (j) MMP9 protein level as compared to phosphate-buffered saline- and L1-treated cells. All these effects of L5 in siC-transfected cells were reversed in LOX1 small interfering ribonucleic acid-transfected HASMCs under L5 stimulation. All results were presented as the mean ± standard error. * $P < 0.05$ versus Ctl- and L1-treated siC group; $^{\#}P < 0.05$ versus L5-treated siC group.

atherosclerosis in L5-stimulated aortic cells. We found that *crbp1* gene transfection could significantly reverse L5 treatment-induced increase of LOX1 (Figure 4a,b), suppress STRA6, CRBP1, RARα and RXRα (Figure 4a,c–g), and increase p-p38, pSmad2, caspase 3 and MMP9 expression in L5-stimulated HAECs (Figure 4a,h–j).

DISCUSSION

In the present study, we showed the mechanism that L5 from MetS patients suppresses the STRA6–CRBP1–LRAT–RARα–RXRα cascade through activating LOX1 in the aortas of L5-injected mice, and L5-treated endothelial and smooth muscle cells. In particular, this study also showed that the L5-suppressed STRA6 signals participated in the process of arteriosclerosis, whereas L1 did not.

An important study showed that the STRA6-deficient zebrafish model is manifested by several developmental malformations, including cardiac defects[21]. The authors showed that STRA6 and LRAT deficiency, and accumulated RBP4-bound retinol, together led to these developmental abnormalities. However, very few studies have illustrated the changes of the STRA6–retinol–RAR cascade in animal or human diseases since

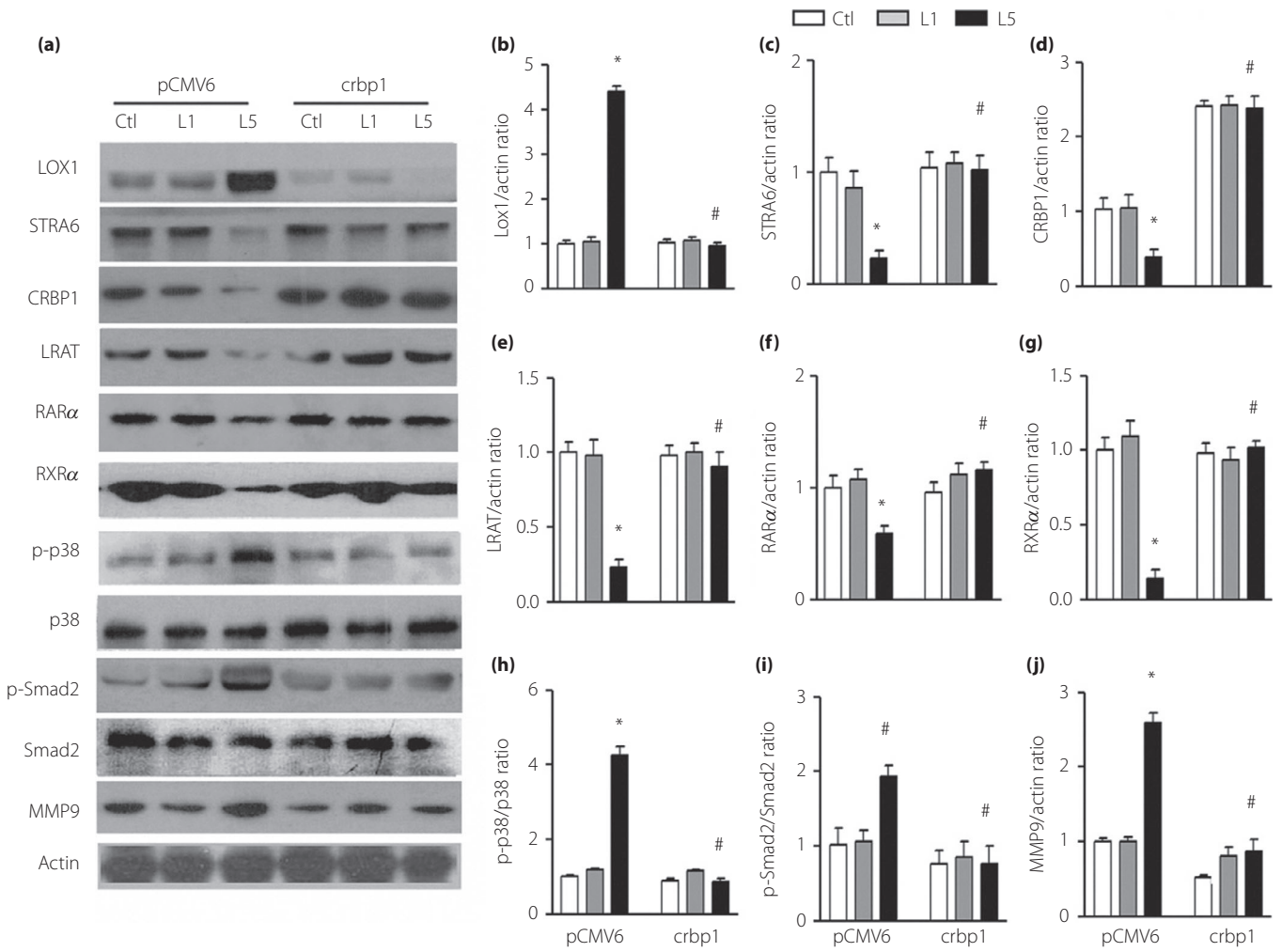

Figure 4 | The *crbp1* gene transfection reverses electronegative low-density lipoprotein (L5) effects on stimulated by retinoic acid 6 (STRA6) cascades and markers of atherosclerosis in human aortic endothelial cell lines (HAECs). (a) Western blots showed LOX1, STRA6, cellular retinol-binding protein 1 (CRBP1), lecithin-retinol acyltransferase (LRAT), retinoic acid receptor (RAR)α, retinoid X receptor (RXR), p38 mitogen-activated protein kinases (p-38), pSmad2 and matrix metallopeptidase 9 (MMP9) expression in cell lysate of pCMV6-transfected and pCMV6-crbp1-transfected HAECs under phosphate-buffered saline (control [Ctl]), L1 and L5 treatment for 24 h ($n = 3$). Quantitative analysis showed significant differences for (b) the increase of LOX1; the decrease of (c) STRA6, (d) CRBP1, (e) LRAT, (f) RARα and (g) RXRα; the increase of (h) p-p38/p38 and (i) pSmad2/Smad2 ratios, and (j) MMP9 under L5 stimulation in pCMV6-transfected cells. These changes caused by L5 treatment were reversed in pCMV6-crbp1-transfected cells. All results are presented as the mean ± standard error. $^{*}P < 0.05$ versus Ctl- and L1-treated plasmid DNA of cytomegalovirus 6 (pCMV6) groups; $^{\#}P < 0.05$ versus L5-treated pCMV6 group.

STRA6 was identified. Recently, the tissue levels of retinol, RA, CRBP-I, RARα, RARβ and RARγ concentrations were reported to decrease in the pancreas, kidney and liver of obesity-related mice[29]. Another study also showed that high glucose could repress RAR/RXR in cardiomyocytes[42]. More recently, the present authors showed that STRA6, CRBP-I, LRAT, RARα, RARβ and RARγ were suppressed in the kidneys of L5-injected mice[37]. These findings might explain why exogenous administration of RA and RARβ2 agonist could inhibit proliferation, migration, differentiation, inflammation, changes of intracellular matrix in arterial smooth muscle cells or in high-fat diet-induced atherosclerosis[31–34,43,44]. This current study is the first to confirm that L5 isolated from MetS patients could suppress the STRA6–CRBP1–LRAT–RARα–RXRα cascade in the aortas of L5-injected mice, but not in L1-injected mice.

LOX1 has been known as an important receptor to be associated with atherosclerotic disease[45,46]. In early atherosclerotic lesions, the increase of LOX1 in endothelial cells and vascular smooth muscle cells can promote expression of adhesion molecules and endothelial dysfunction[47,48]. Our previous study showed that the elevation of L5 is associated with endothelial dysfunction, including apoptosis and platelet aggregation

through LOX1[11,13]. More recently, our study showed that LOX activation could significantly repress STRA6 cascades in the kidney[37]. The current study showed that L5 isolated from MetS patients activates LOX1 expression while concomitantly disrupting the STRA6–CRBP1–retinol–LRAT–RARα–RXRα pathway, and increases atherosclerotic makers including TGFβ1, active caspase 3 and MMP9 in the aortas of L5-injected mice, as compared with L1-injected mice. The suppression of STRA6–retinol signals and the increase of atherosclerotic makers do not appear in aortas of L5-injected LOX1$^{-/-}$ mice. Furthermore, LOX1 gene silencing recovers the suppression of STRA6, CRBP1, retinol, LRAT, RARα and RXRα in L5-treated aortic endothelial cells and smooth muscle cells. These results elucidate that native L5 from MetS patients causes the disruption of STRA6 cascades and pathogenesis of aorta through activation of LOX1.

In the atherosclerotic process, many component pathways are involved, such as TGFβ$_1$–Smad signaling and p38MAPK pathways, and lead to matrix formation, stenosis and endothelial-to-mesenchymal transition[38–40,49–,51]. MMP9 is secreted from vascular cells and macrophage foamy cells, and participates in several stages of atherosclerosis, including monocyte recruitment, endothelial cell dysfunction, SMC migration, fibrous cap formation and destabilization of the plaque[41,52,53]. In clinical settings, the circulating MMP9 levels were increased in individuals with coronary artery disease[54], and particularly in individuals with MetS[55]. Inside the blood vessels, the macrophage foam cells and smooth muscle cells can secrete a large amount of MMP9 in response to oxidized LDL, reactive oxygen species and several inflammatory factors[51–56]. Here, the present study showed that native L5 isolated from MetS patients could markedly increase atherogenic markers (TGFβ$_1$, phosphorylation of p38MAPK and Smad2, MMP9 and caspase 3) in the aortas of C57B6/J mice, but L1 did not. These alterations induced by L5 in the aortas of control mice were reversed in LOX1$^{-/-}$ mice. Additionally, L5 treatment also increased MMP9, phosphorylation of p38MAPK and Smad2 in HAECs and HASMCs, but L1 did not. The increase of MMP9, phosphorylation of p38MAPK and Smad2 induced by L5 in vascular cells was reversed by LOX1 silencing. Altogether, the present study provides new evidence that native L5 from MetS patients can cause atherosclerotic formation.

STRA6-mediated retinol delivery into cells requires CRBP1, an intracellular retinol acceptor[21]. A recent study reported that retinol deficiency could promote atherogenesis in apolipoprotein E knockout mice[57]. In a previous study, we found *crbp1* gene transcription could reverse L5-damaged STRA6 signaling of renal cells[37]. The STRA6 signaling pathway could be recovered under *crbp1* transfection, and the L5-induced p38MAPK phosphorylation, TGFβ elevation, Smad2 phosphorylation, apoptosis and fibrosis were significantly repressed in renal tubular cells[37]. It means that L5 disrupts STRA6 signaling and strongly causes cell damage. Therefore, we predict that the recovery of CRBP1 could ameliorate retinol transport into cells from STRA6, and we accordingly carried out experiments of *crbp1* gene transfection. Our results showed that the L5-induced suppression of STRA6, LRAT, RARα and RXRα was retrieved, and the L5-induced activation of MMP9, phosphorylation of p38MAPK and Smad2 were attenuated by *crbp1* gene transfection in L5-treated endothelial cells. These results imply that L5-mediated suppression of STRA6/CRBP1/RARαs/RXRα might participate in atherosclerosis mediated by L5 from patients with MetS.

In conclusion, we showed that electronegative L5 from MetS patients can increase atherosclerotic markers, and simultaneously suppress STRA6, CRBP1, RARα and RXRα cascades. The alteration of the STRA6 cascade might be involved in atherosclerotic formation caused by L5 treatment. The present study implies that the suppression of STRA6 signals might participate in MetS-related arterial damage.

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DISCLOSURE

The authors declare no conflict of interest.

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