Original Article

Pitavastatin Increases HDL Particles Functionally Preserved with Cholesterol Efflux Capacity and Antioxidative Actions in Dyslipidemic Patients

Maki Miyamoto-Sasaki¹, Tomoyuki Yasuda¹, Tomoko Monguchi¹, Hideto Nakajima¹, Kenta Mori¹, Ryuji Toh^{1,2}, Tatsuro Ishida¹ and Ken-ichi Hirata¹

¹Division of Cardiovascular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

²Division of Evidence-based Laboratory Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

Aim: Although statins increase the plasma concentration of high-density lipoprotein cholesterol (HDL-C), it has not been elucidated whether the increased HDL particles possess normal antiatherosclerotic properties. Pitavastatin functions to increase the plasma HDL-C level and decrease the low-density lipoprotein cholesterol (LDL-C) level. In the present study, we sought to examine the qualitative changes in HDL during pitavastatin treatment.

Methods: A total of 30 patients with dyslipidemia were treated with 2 mg of pitavastatin for four weeks. The cholesterol efflux capacity and activities of the antioxidative enzymes paraoxonase-1 (PON-1) and platelet-activating factor acetylhydrolase (PAF-AH) were evaluated using polyethethylene glycol-treated HDL fractions before and after pitavastatin treatment.

Results: Pitavastatin treatment decreased the serum LDL-C level by 39% and increased the serum HDL-C level by 9% ($p < 0.05$). In addition, pitavastatin increased the phospholipid content of HDL by 7.8% ($p < 0.05$). The pitavastatin-induced increase in the HDL-C level coincided with an increase in the cholesterol efflux capacity of the isolated HDL fraction of 8.6% ($p < 0.05$). The post-pitavastatin treatment activity of HDL-associated PON-1 (paraoxonase and arylesterase) was increased by 9% ($p < 0.05$) and 11% ($p < 0.05$), respectively, while the HDL-associated PAF-AH activity was not affected by pitavastatin.

Conclusions: In addition to its LDL-C-lowering effects, pitavastatin elevates the HDL-C level and enhances the cholesterol efflux capacity and antioxidative properties of HDL. Pitavastatin therefore increases the amount of functional HDL without attenuating HDL quality.

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Key words: Statin, HDL, Cholesterol efflux, PON-1

Introduction

It has been established that low-density lipoprotein cholesterol (LDL-C) is a central therapeutic target for coronary artery disease (CAD)¹⁾. Although statins reduce the risk of CAD by 30-40%, cardiovascular

events, known as “residual risks”²⁾, can continue to occur during intensive LDL-lowering therapy, and research aims to identify new therapeutic targets beyond LDL-C. The well-characterized residual risks following statin treatment include a high level of triglycerides (TGs), a low level of high-density lipoprotein cholesterol (HDL-C), uncontrolled diabetes mellitus, hypertension, obesity and lifestyle factors, such as smoking and inactivity. Such factors are not controlled with statin treatment. There is an inverse correlation between the circulating HDL-C level and the risk of CAD³⁾. Even after statin treatment, the inverse correlation between the incidence of cardiovascular

Address for correspondence: Tomoyuki Yasuda, Division of Cardiovascular Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

E-mail: toyasuda@med.kobe-u.ac.jp

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events and the circulating HDL-C level still holds⁴). HDL exhibits a variety of cardiovascular protective effects by promoting reverse cholesterol transport (RCT) from the vascular wall to the liver⁵). HDL particles also possess several antiatherogenic functions, such as anti-inflammatory, antiapoptotic, antithrombotic and immunomodulating effects⁶). Therefore, it is believed that HDL-raising therapy can reduce the incidence of cardiovascular events. However, large clinical trials using cholesteryl ester transfer protein (CETP) inhibitors have failed to document the efficacy of HDL-raising therapy, despite a marked increase in the serum HDL-C level and a decrease in the serum LDL-C level⁷). Therefore, both raising the HDL level and improving the quality of functional HDL have become interesting topics of discussion in HDL-targeting therapy. Under pathological conditions, HDL particles have been reported to lose their antiatherogenic function and convert into dysfunctional HDL, which promotes inflammation and oxidation^{8,9}).

The HDL-raising effects of statins are variable in contrast to their comparable LDL-lowering effects¹⁰). Pitavastatin is a unique statin that increases the HDL-C level more significantly than other strong statins¹⁰⁻¹³). Furthermore, pitavastatin improves event-free survival following percutaneous coronary intervention¹³). The mechanisms by which pitavastatin increases the HDL-C level¹⁴⁻¹⁶) are partially understood; however, the effects of pitavastatin on the quality of HDL remain unknown. In this study, we investigated qualitative and quantitative changes in HDL during pitavastatin treatment. To this end, we evaluated the cholesterol efflux capacity of HDL, the first step in RCT, and the antioxidant properties of the paraoxonase-1 (PON-1) and platelet-activating factor acetylhydrolase (PAF-AH) activities.

Materials and Methods

Patients

This investigation conformed to the principles outlined in the Declaration of Helsinki. All patients gave their written informed consent, and the clinical study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine. A total of 30 patients with dyslipidemia, as defined by the Japan Atherosclerosis Society guidelines¹⁷), were enrolled in this study. The backgrounds of the participants are described in **Table 1**. The patients were treated with 2 mg of pitavastatin daily for four weeks, and serum was obtained pre- and post-pitavastatin treatment. Blood samples were collected after overnight fasting, and the serum was stored at -80°C until

Table 1. The background of the enrolled dyslipidemia patients

Variable	Patients with Dyslipidemia (n=30)
Male, n (%)	22 (73.3)
Age (year)	59.6 $\pm$ 15.9
Body mass index (kg/m ²)	24.1 $\pm$ 3.0
Smoking status	
Never smoked, n (%)	18 (60.0)
Former smoker, n(%)	10 (33.3)
Current smoker, n (%)	2 (6.6)
Alcohol consumption	
None, n (%)	14 (46.7)
Moderate, n (%)	13 (43.3)
Excessive, n (%)	3 (10.0)
Past history	
CAD history, n (%)	22 (73.3)
Hypertension, n (%)	10 (33.3)
Diabetes mellitus, n (%)	10 (33.3)
Dyslipidemia, n (%)	30 (100)
Stroke, n (%)	0 (0)
Family history of CAD and CAD risk factor, n (%)	7 (23.3)

CAD; coronary artery disease, data represent mean $\pm$ SD

the analysis.

Preparation of the HDL and LDL Fractions

The serum samples were thawed on ice and incubated with 20% polyethethylene glycol (PEG) (Sigma-Aldrich, MO, USA) solution in 200 mmol/L of glycine buffer to remove apolipoprotein B (apoB)-containing lipoproteins, as previously described¹⁸). In brief, each serum sample was mixed with PEG solution (100:40) and incubated for 15 minutes at room temperature. The samples were then centrifuged at 4,000 rpm for 20 minutes to precipitate all apoB-containing lipoproteins, and the supernatant was kept as the HDL fraction (PEG-HDL). Phosphate-buffered saline (PBS) was incubated with PEG solution and centrifuged according to the same methods as the serum, and the supernatant was used as a control (PEG-PBS). LDL was isolated from pooled human plasma using ultracentrifugation (1.020-1.063)¹⁹). Acetyl LDL was generated by incubating LDL with acetic anhydride²⁰), then used in the cholesterol efflux assay.

Lipid Analysis

The serum lipid levels were measured enzymatically using a commercially available kit from WAKO (Osaka, Japan). The serum and HDL-associated apolipoprotein A-I (apoA-I), apoA-II and apoE levels were

Table 2. Serum Lipid profile of pre- and post-pitavastatin treatment

Variable	Patients with dyslipidemia (<i>n</i> =30) pre	Patients with dyslipidemia (<i>n</i> =30) post	<i>p</i> value
Serum concentration Cholesterol (mg/dL)			
Total-C(mg/dL)	224.4 ± 40.1	165.5 ± 28.4	≤ 0.01
HDL-C (mg/dL)	50.7 ± 11.4	55.3 ± 11.4	≤ 0.01
LDL-C (mg/dL)	146.3 ± 31.7	89.6 ± 18.6	≤ 0.01
Triglycerides (mg/dL)	152.7 ± 100.3	123.7 ± 64.2	0.02
Median	118	109	
Interquartile range	60-454	46-313	
Apolipoprotein (mg/dL)			
apoA-I (mg/dL)	120.0 ± 31.5	124.4 ± 35.5	0.16
Phospholipid (mg/dL)			
Total-PL (mg/dL)	190.9 ± 30.3	168.6 ± 22.3	≤ 0.01
HDL fraction			
apoA-I (mg/dL)	69.3 ± 24.8	73.4 ± 31.2	0.52
apoA-II (mg/dL)	13.0 ± 7.3	12.4 ± 4.2	0.67
apoE (mg/dL)	1.82 ± 1.21	1.25 ± 0.57	0.053
HDL-PL (mg/dL)	117.3 ± 43.6	145.2 ± 58.6	0.047
FC/CE ratio	0.17 ± 0.06	0.17 ± 0.05	0.75

Total-C; total-cholesterol, HDL-C; high density lipoprotein-cholesterol, LDL-C; low density lipoproteincholesterol, apoA-I; apolipoprotein A-I, PL; phospholipid, FC; free cholesterol, CE; cholesteryl ester, HDL fraction was purified with polyethylene glycol precipitation. data represent mean ± SD

measured using a commercially available ELISA kit (ASSAYPRO, MO, USA). The lipoprotein profiles were determined using HPLC (LipoSEARCH[®]) at Skylight Biotech, Inc. (Akita, Japan), according to the specified procedure²¹. The levels of HDL-phospholipids, total cholesterol (TC), and free cholesterol (FC) were measured with PEG-HDL using a commercially available kit from WAKO (Osaka, Japan). The level of HDL-cholesteryl ester (CE) was calculated by subtracting the level of HDL-FC from the level of HDL-TC.

Cholesterol Efflux Study

The cholesterol efflux studies were performed as previously described¹⁸. The human monocyte cell line THP-1 (RIKEN, Tsukuba, Japan) was cultured in RPMI 1640 medium (Wako, Osaka, Japan) containing 10% fetal bovine serum. The cells were harvested in 24-well plates and differentiated into macrophages using 200 nmol/L of phorbol myristate acetate (Sigma-Aldrich, MO, USA) for 72 hours. Next, the adherent macrophages were incubated with 25 µg/mL of acetyl LDL and 1 µCi of [³H] cholesterol (Perkin-Elmer Life Science, MA, USA) for 24 hours, followed by treatment with 10 µmol/L of Liver X receptor agonist TO901317 (Sigma-Aldrich, MO, USA) for 16 hours. The cells were then incubated with either 2.8% PEG-HDL or PEG-PBS in RPMI 1640 medium with

0.2% bovine serum albumin for four hours to induce cholesterol efflux. The percent efflux capacity was calculated according to the following formula: (DPM of [³H] cholesterol in media containing 2.8% PEG-HDL) ÷ [(DPM of [³H] cholesterol in media containing 2.8% PEG-HDL) + (DPM of [³H] cholesterol in cells extracted after efflux study)] × 100. The PEG-HDL-specific efflux capacity was calculated according to the following formula: [(% cholesterol efflux by 2.8% PEG-HDL) - (% cholesterol efflux by PEG-PBS)].

PON-1 and PAF-AH Activities

The PON-1 activity was analyzed spectrophotometrically with PEG-HDL (HDL-associated PON-1), as previously reported²². Two individual PON-1 activities were evaluated. The paraoxonase activity was measured with paraoxon (Sigma-Aldrich, MO, USA) as a substrate, whereas the arylesterase activity was measured with phenylacetate (Sigma-Aldrich, MO, USA) as a substrate. The HDL-associated PAF-AH activity was measured using a commercially available kit (Cayman Chemicals, MI, USA).

Statistical Analysis

All values are presented as the mean ± SD. Comparisons between the parameters of pre- and post-pitavastatin treatment were made with paired Stu-

dent's *t*-test (two-tailed) using the GraphPad Prism Software program.

Results

Pitavastatin Increased the Serum HDL-C and HDL-Phospholipids Levels

The lipid profiles of the patients (Table 1) pre- and post-pitavastatin treatment are shown in Table 2. Pitavastatin significantly decreased the serum Total-C, LDL-C and TG levels and increased the serum HDL-C levels in the dyslipidemic patients by 9% compared to the baseline values. Pitavastatin treatment led to a significant increase in the phospholipid content of the HDL fraction of 7.8%. The HPLC analysis revealed that the peak HDL fraction deviated to the left, indicating an increase in the number of large HDL particles (Fig. 1A, B). The levels of ApoA-I, apoA-II and apoE and the FC/CE ratio of HDL did not change during pitavastatin treatment. Taken together, pitavastatin increased the number of phospholipid-rich large HDL particles without modifying the apolipoprotein distribution.

Pitavastatin Increased the Serum Cholesterol Efflux Capacity

The cholesterol efflux capacity of HDL isolated via PEG precipitation was performed using THP-1 macrophages incubated with acetyl LDL and the Liver X receptor agonist in order to mimic the macrophage phenotypes in atherosclerotic lesions. We confirmed that TO901317 treatment increased the mRNA expressions of ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1), while acetyl LDL treatment decreased the scavenger receptor class B type I (SR-BI) mRNA expression in THP-1 macrophages (data not shown). Pitavastatin significantly increased the PEG-HDL-specific cholesterol efflux capacity in the dyslipidemic patients by 8.6% (6.6 ± 1.4 vs $7.1 \pm 1.7\%$; $p < 0.05$) (Fig. 2A, 2B). The cholesterol efflux capacity standardized according to the serum apoA-I level was increased during pitavastatin treatment (Fig. 2), indicating that the increased HDL level contributed to the increased efflux capacity. These findings suggest that pitavastatin increases the amount of HDL particles that are functionally preserved with a cholesterol efflux capacity.

Pitavastatin Enhanced Serum Antioxidant Properties

The PON-1 activity is widely used to evaluate the antioxidant properties of HDL^{6, 23, 24}. Therefore, the HDL-associated PON-1 activities in the pre- and post-pitavastatin serum samples were measured. The

Fig. 1. Effects of pitavastatin treatment on lipoprotein profiles evaluated using HPLC.

The serum samples of dyslipidemic patients were subjected to HPLC. Fractions 1,2: Chylomicron remnant, 3-7: very-low-density lipoprotein, 8-13: low-density lipoprotein, 14-20: high-density lipoprotein A. HPLC profiles of the cholesterol levels (mg/dL). B. HPLC profiles of the triglyceride levels (mg/dL).

HDL-associated paraoxonase activity was increased in the dyslipidemic patients by 9% (23.2 ± 9.7 vs 25.0 ± 10.5 pmol/min/mL PEG-HDL; $p < 0.05$) (Fig. 3A, 3B). The HDL-associated arylesterase activity was increased by 11% (58.7 ± 20 vs 63.1 ± 19 pmol/min/mL PEG-HDL; $p < 0.05$) (Fig. 3C, 3D). Neither the HDL-associated paraoxonase or arylesterase activity standardized according to the serum apoA-I level changed during pitavastatin treatment (data not shown). Similarly, the HDL-associated PAF-AH activity did not change during pitavastatin treatment (71.5 ± 21.2 vs 71.2 ± 25.6 pmol/min/mL PEG-HDL; $p = 0.92$) (Fig. 4A, 4B). These data suggest that pitavastatin enhances serum antioxidant properties by increasing the number of HDL particles that possess PON-1 activities.

Fig. 2. Effects of pitavastatin treatment on PEG-HDL-specific cholesterol efflux in the dyslipidemic patients.

A. The cholesterol efflux capacity induced by the same volume of 2.8% PEG-HDL in TO901317-treated THP-1 macrophages pre- and post-pitavastatin treatment. B. The percent change in the PEG-HDL-specific cholesterol efflux capacity pre- and post-pitavastatin treatment. C. The cholesterol efflux induced by the same volume of 2.8% PEG-HDL standardized according to the serum apoA-I level pre- and post-pitavastatin treatment. The bars represent the mean $\pm$ SEM. * $p < 0.05$

Discussion

Although a large number of epidemiological studies have documented a strong inverse association between the plasma HDL-C level and the incidence of cardiovascular events, the benefits of HDL-raising pharmaceutical intervention remain controversial^{7, 25}. The relationship between the plasma HDL-C level and the risk of cardiovascular events is often explained by the functional quality of HDL, including cholesterol efflux and the antioxidative, anti-inflammatory and antiproliferative properties⁶. Cholesterol efflux from macrophages is the rate-limiting first step in RCT from a diseased vessel wall to the liver. A recent study demonstrated that the cholesterol efflux capacity of HDL is an independent negative risk factor for CAD²⁶. Additionally, Patel *et al.* reported that the

antioxidative capacity of HDL, as evaluated in a cell-free assay, is attenuated in patients with acute coronary syndrome²⁷. Therefore, changes in the quality of functional HDL are closely related to the risk of cardiovascular disease. In this study, pitavastatin treatment increased the cholesterol efflux capacity and the PON-1 activity in association with antioxidant properties in conditioned HDL isolated from dyslipidemic patients. These effects occurred in parallel with an increase in the serum HDL-C level. Taken together, pitavastatin increased the amount of functional HDL without attenuating the HDL quality.

Several plausible explanations have been proposed for the molecular mechanisms underlying the effects of pitavastatin on HDL. Previous studies have indicated that pitavastatin increases apoA-I production both *in vitro*¹⁴ and *in vivo*²⁸. In addition,

Fig. 3. Effects of pitavastatin treatment on the paraoxonase activity in the dyslipidemic patients.

A. The paraoxonase activity of the same volume of PEG-HDL (=HDL-associated paraoxonase activity) pre- and post-pitavastatin treatment. B. The percent change in the paraoxonase activity pre- and post-pitavastatin treatment. C. The arylesterase activity of the same volume of PEG-HDL (=HDL-associated arylesterase activity) pre- and post-pitavastatin treatment. D. The percent change in the arylesterase activity pre- and post-pitavastatin treatment. The bars represent the mean $\pm$ SEM. * $p < 0.05$

pitavastatin has been reported to increase the ABCA1 expression *in vitro*²⁹). Pitavastatin also decreases the expression of endothelial lipase, which increases not only the quantity of HDL, but also the HDL particle size by increasing the amount of phospholipid content¹⁶). The phospholipid-rich HDL particles generated by EL suppression exert anti-inflammatory and antioxidative effects²²). As for cholesterol efflux, in the macrophages used in the efflux method in this study, both ABCA1 and ABCG1 were upregulated by the LXR agonist, and SR-BI was downregulated by acetyl LDL treatment. The macrophages ABCA1 and ABCG1 play crucial roles in *in vivo* reverse cholesterol transport³⁰). ABCA1 primarily promotes cholesterol efflux through the actions of lipid-poor apoA-I and nascent HDL, whereas ABCG1 and SR-BI mediate efflux

through the actions of mature HDL³¹). Given that pitavastatin increases the amount of mature phospholipid-rich HDL and decreases the amount of nascent pre-beta HDL¹⁵), we speculate that the increased level of mature HDL induced by pitavastatin contributes to enhancing the efflux capacity, most likely via the actions of ABCG1. In brief, pitavastatin increases the amount of HDL via a dual mechanism through which it attenuates HDL catabolism and promotes HDL synthesis.

Khera *et al.* recently reported that statin treatment did not increase the serum cholesterol efflux capacity in their cohort study²⁶). However, the serum cholesterol efflux capacity was measured at only one time point and pitavastatin was not included in the medication regimen. Pitavastatin has a greater capacity

Fig. 4. Effects of pitavastatin treatment on the PAF-AH activity in the dyslipidemic patients.

A. The PAF-AH activity of the same volume of PEG-HDL (=HDL-associated PAF-AH activity) pre- and post-pitavastatin treatment. B. The percent change in the HDL-associated PAF-AH activity pre- and post-pitavastatin treatment. The bars represent the mean $\pm$ SEM.

to increase the HDL-C level than other statins¹³⁾, and the present study directly assessed changes in the efflux capacity pre- and post-pitavastatin treatment.

PON-1 is an HDL-associated protein that exerts antioxidative effects on HDL. The PON-1 activity is evaluated using several individual substrates. The substrate of the paraoxonase activity assay is paraoxon, while that of the arylesterase activity assay is phenylacetate, and there is a positive relationship between the results of these assays²²⁾. Previous studies have shown that statins increase the PON-1 mass and activity by up to 40%³²⁾. In the present study, pitavastatin increased the HDL associated-paraoxonase activity by 10% and the HDL associated-arylesterase activity by 14%, which dominated the increased serum HDL-C level. This finding may be partially due to the fact that pitavastatin directly upregulates the PON-1 expression in cultured hepatocytes³³⁾.

In this study, we selected pitavastatin because it has a greater capacity to raise the HDL level than other statins; however, the HDL-raising effects of pitavastatin are shared by other statins¹⁰⁾. Clinical studies of both pravastatin³⁴⁾ and rosuvastatin³⁵⁾ have shown that regression of the atherosclerotic plaque volume is positively correlated with changes in the HDL-C level. Combining these clinical data with our results, the raised HDL level observed during statin therapy may be beneficial for reducing the amount of atherosclerotic lesions by increasing the cholesterol efflux capacity and antioxidant properties.

It has not been elucidated yet whether the statin-induced changes observed in the HDL antiatherogenic properties are physiologically relevant to preventing

the progression of atherosclerosis, although both the cholesterol efflux capacity and the PON-1 activity are correlated with the prevalence of atherosclerosis^{23, 26)}. In the present study, changes in the cholesterol efflux capacity were not correlated with changes in the PON-1 activity (data not shown). Furthermore, the activity of another antioxidant enzyme, PAF-AH, was not affected by pitavastatin. Previous reports have also shown that the cholesterol efflux capacity is not always associated with antioxidant properties^{26, 27)}. These findings indicate that each parameter representing HDL antiatherosclerotic properties is independent, and further studies are needed to identify the most important and relevant markers of the quality of functional HDL.

The results of clinical trials of CETP inhibitors are confusing with respect to HDL-raising therapies. Two CETP inhibitors, torcetrapib⁷⁾ and dalcetrapib³⁶⁾, failed to reduce the incidence of cardiovascular events despite raising the HDL-C levels. It has been reported that the HDL elevated by CETP inhibition or deficiency possesses normal or enhanced cholesterol efflux³⁷⁾ and antioxidant properties³⁸⁾, indicating that dysfunctional HDL does not affect the overall results. In general, statins inhibit the CETP activity¹⁰⁾, and the increased HDL level induced by statins may be partially due to CETP inhibition. Therefore, both statins and CETP inhibitors increase the amount of functionally preserved HDL. However, there is a large discrepancy in clinical outcomes between statins and CETP inhibitors, the precise mechanisms of which remain unknown. These data imply that raising the level of functional HDL via pharmaceutical intervention alone does not lead to improved clinical out-

comes in patients with cardiovascular disease. Clinical studies of two other novel CETP inhibitors are currently ongoing, the results of which may provide additional insight.

Several limitations are associated with the present study. This study consisted of a small number of subjects, and we did not compare the effects of pitavastatin with those of other statins. In addition, the duration of pitavastatin treatment was not long, although several clinical studies have shown that pitavastatin treatment increases the HDL level by over 10% for longer periods^{12, 16}. In our study cohort, few participants exhibited low levels of HDL-C, and the effects of statins may have been greater if the study was conducted among low HDL-C patients only. Further studies should be performed to evaluate these issues.

In conclusion, pitavastatin treatment increases the serum HDL-C levels in association with increases in the cholesterol efflux capacity and PON-1 activity in dyslipidemic patients. In general, pitavastatin increases the amount of HDL particles while functionally preserving antiatherosclerotic properties. The increased quantity and quality of HDL may at least, in part, contribute to the antiatherosclerotic effects of this statin.

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Abbreviations

HDL-C; high density lipoprotein, LDL; low density lipoprotein, TG; triglyceride, CETP; cholesteryl ester transfer protein, PON-1; paraoxonase-1, PAF-AH; platelet-activating factor acetylhydrolase, RCT; reverse cholesterol transport, CAD; coronary artery disease, apoA-I; apolipoprotein A-I, apoA-II; apolipoprotein A-II, apoB; apolipoprotein B, ABCA1; ATP-binding cassette transporter A1, ABCG1; ATP-binding cassette transporter G1, SR-BI; scavenger receptor class B type I, PEG; polyethethylene glycol, EL; endothelial lipase

Conflicts of Interest

None.

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