

Statin-Induced Decrease in ATP-Binding Cassette Transporter A1 Expression via microRNA33 Induction may Counteract Cholesterol Efflux to High-Density Lipoprotein

Eric J. Niesor · Gregory G. Schwartz · Anne Perez ·
Andrea Stauffer · Alexandre Durrwell ·
Gabriela Bucklar-Suchankova · Renée Benghozi ·
Markus Abt · David Kallend

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Abstract

Purpose Cholesterol efflux from macrophages to HDL, measured in vitro, is augmented by treatment with agents which raise HDL cholesterol. In vitro, cholesterol depletion by statins is known to trigger a positive feedback on the cholesterol synthetic pathway via sterol regulatory element-binding protein (SREBP) transcription and changes in expression of SREBP regulated genes including microRNA33 (miR33) which is co-transcribed with SREBP and down-regulates ABCA1 and ABCG1 expression.

Methods We investigated whether miR33 up-regulation, associated with SREBP increased transcription by statins, reduces macrophage ATP-binding cassette (ABC) transporter expression, thereby decreasing HDL-mediated cholesterol efflux at the tissue level.

Results In human macrophage THP-1 cells cholesterol-loaded with acetylated LDL, incubation with 1 μM

atorvastatin increased miR33 by 33 % ($P < 0.05$), and decreased ABCA1 messenger RNA (mRNA) and ABCG1 mRNA by 47 % ($P < 0.05$) and 27 % (NS), respectively. In J774A.1 mouse macrophage, labelled with 3H-cholesterol, ABCA1 mRNA and ABCA1-mediated cholesterol efflux were decreased by 1 μM statin: simvastatin > pitavastatin > atorvastatin > rosuvastatin > pravastatin. HDL incubated with rhCETP and dalcetrapib increased ABCA1-mediated cholesterol efflux. However, incremental simvastatin concentrations decreased cholesterol efflux to HDL treated with rhCETP and dalcetrapib. When HDL was incubated with rhCETP, addition of dalcetrapib augmented ABCA1-mediated cholesterol efflux from J774A.1 macrophages. However, simvastatin $\geq 1 \mu\text{M}$ virtually eliminated any HDL-ABCA1-mediated cholesterol efflux and any augmentation of that process by dalcetrapib.

Conclusions In vitro, statins increase miR33 expression, and decrease ABCA1 expression and cholesterol efflux from peripheral tissues; this may counteract the potential benefit of agents that raise HDL and apolipoprotein A-I in statin-treated patients.

E. J. Niesor (✉) · A. Perez · A. Stauffer · A. Durrwell
Pharma Research & Early Development, pRED, F. Hoffmann-La Roche Ltd, Basel, Switzerland
e-mail: eric_j.niesor@roche.com

G. G. Schwartz
Veterans Affairs Medical Center and University of Colorado School of Medicine, Denver, CO, USA

R. Benghozi · D. Kallend
Pharma Development, F. Hoffmann-La Roche Ltd,
Basel, Switzerland

M. Abt
Biostatistics, F. Hoffmann-La Roche Ltd, Basel, Switzerland

G. Bucklar-Suchankova
Roche Professional Diagnostics, Basel, Switzerland

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Introduction

The strong, inverse relationship between HDL cholesterol (HDL-C) and cardiovascular risk, mainly from populations untreated with lipid-modifying agents [1], is attributed to the role of HDL in reverse cholesterol transport (RCT) [2]. Most

patients at elevated cardiovascular risk are treated with a statin and, while controversial from individual studies, large meta-analyses suggest low HDL-C still represents a cardiovascular risk factor on a background of statin treatment [3]. However, not all published data support this. The JUPITER trial compared 20 mg rosuvastatin with placebo for primary prevention of cardiovascular events in patients with elevated C-reactive protein. Median baseline HDL-C was ~51 mg/dl and did not change during the trial. HDL-C and apolipoprotein (Apo) A-I levels were strongly related to risk of major cardiovascular events in the placebo group but not in the rosuvastatin group [4]. The dal-OUTCOMES trial compared the cholesteryl ester transfer protein (CETP) modulator dalcetrapib [5] with placebo in nearly 16,000 patients with acute coronary syndrome; 97 % of whom were statin-treated with a mean baseline LDL cholesterol (LDL-C) of 76 mg/dl [6]. There was no relationship between baseline HDL-C or ApoA-1 levels and risk of major cardiovascular events for either treatment arm. Dalcetrapib increased HDL-C and ApoA-1 concentrations by 31 % and 11 %, respectively, with minimal effect on LDL-C and no effect on cardiovascular risk. Two trials have compared niacin with placebo, added to simvastatin [7, 8] (mean baseline HDL-C 35 and 44 mg/dl, respectively) and, although niacin raised HDL-C by ~15 %, there was no clinical benefit. Collectively, these observations suggest that, against a background of statin treatment, there may be no relationship of HDL-C to risk and no clinical benefit of raising HDL-C.

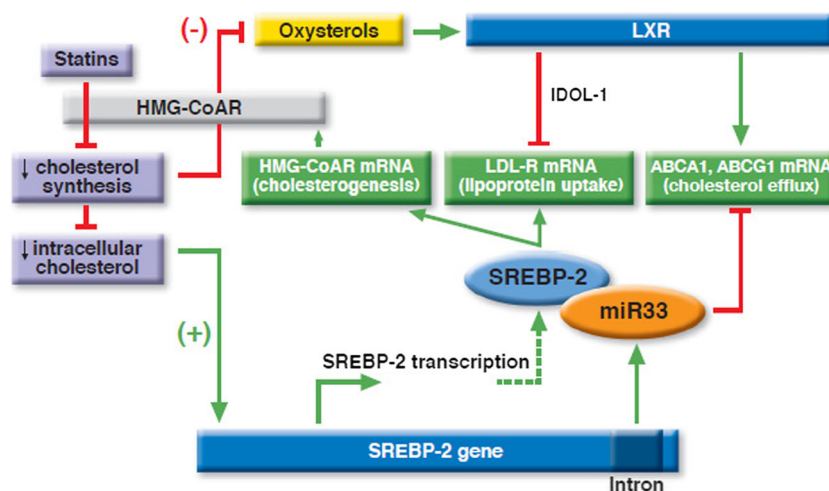
ATP-binding cassette (ABC) transporters mediate intracellular cholesterol transfer to HDL and are rate-limiting for RCT [9, 10]. Several cellular and plasma components are involved in the complex process of RCT and their role as well as their relative importance was reviewed by Rosenson et al. [11]. The cholesterol circulating in HDL originates almost exclusively from ABCA1 since, in the absence of ABCA1 [12] or decreased expression and function of ABCA1 [13], plasma levels of HDL-C are proportionally affected.

There are at least two potential mechanisms by which statins might reduce expression of the ABC transporters ABCA1 and ABCG1. The membrane cholesterol level is tightly regulated, and decreasing intracellular cholesterol concentration with statins increases the transcription of sterol regulatory element binding protein 2 (SREBP-2) to counteract the decrease in cholesterol and limit further elimination [14]. MicroRNA33 (miR33) is transcribed simultaneously and down-regulates the expression and decrease of cholesterol and phospholipid efflux transporters ABCA1 and/or ABCG1 expression [15]. The second mechanism involves the decrease by statin of oxysterols [16], which are ligands of liver X receptor (LXR) transcription factor regulating the expression of ABCA1 and ABCG1 [17]. These two mechanisms may concur to prevent excess removal of cholesterol from the cell membrane. They are shown schematically in Fig. 1.

Although the *in vivo* significance is not yet fully established, it should be noted that statins are known to differentially affect liver and peripheral tissue ABCA1 expression *in vitro* (reviewed by Sato [18]); these observations merit further investigation, especially for therapeutic interventions targeting cholesterol efflux mechanisms. Several reports have shown that miR33 regulates cholesterol efflux and HDL biogenesis by down-regulating ABCA1 and ABCG1 [19–21]; in addition Horie et al. [15] have reported both a time and dose-dependent decrease in ABCA1 protein in simvastatin treated THP-1.

Cholesterol efflux capacity of HDL *in vitro* is usually assessed by incubating samples with macrophage cell lines pre-loaded with labelled cholesterol, but not exposed to statin. Accordingly, if statin exposure of macrophages *in vivo* reduces their expression of ABC transporters and their capacity for cholesterol efflux to HDL, such an effect would not be identified using standard *in vitro* techniques.

Fig. 1 Unified paradigm for cholesterol homeostasis. ABC, ATP-binding cassette transporter; HMG-CoAR, 3-hydroxy-3-methylglutaryl-CoA reductase; IDOL-1, inducible degrader of the LDL-R; LDL-R, LDL receptor; LXR, liver X receptor; miR33, microRNA33; mRNA, messenger RNA; SREBP-2, sterol regulatory element binding protein 2. Adapted from Marquart TJ et al. Proc Natl Acad Sci USA. 2010



We hypothesized that statins increase miR33 expression and reduce ABC transporter expression, thereby reducing the first step of RCT from peripheral tissues and possibly impairing the *in vivo* tissue cholesterol efflux expected from dalcetrapib-induced HDL remodeling.

Methods

In vitro Investigations

Cells

The human acute monocytic leukemia (THP-1) cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, 10 % FBS, 50 μM β -mercaptoethanol, 10 U/ml penicillin, and 10 $\mu\text{g}/\text{ml}$ streptomycin. J774A.1 mouse macrophages were cultured in DMEM containing 1 g/l D-glucose, 10 % FBS, and antibiotics as above. THP-1 cells were seeded to 100,000 cells/100 μl culture medium containing 100 nM phorbol-12-myristate-13-acetate and J774A.1 cells to 40,000 cells/100 μl culture medium, in 96-well plates. Cholesterol loading was performed the next day by incubation for 24 h with 4 $\mu\text{Ci}/\text{ml}$ ^3H -cholesterol and acetylated LDL (acLDL) (acLDL: 50 $\mu\text{g}/\text{ml}$ THP-1, 25 $\mu\text{g}/\text{ml}$ J774A.1). All analyses were performed in triplicate.

Expression of miR33, ABCA1, ABCG1, and LDL receptor messenger RNA, in THP-1 cells Cholesterol-loaded THP-1 cells were incubated with atorvastatin (1 μM) or simvastatin (0.1, 1 and 10 μM) for 24 h. Gene expression analysis was performed either by quantitative polymerase chain reaction or using branched DNA technology (QuantiGene 2.0, Panomics).

Cholesterol Efflux

THP-1 cells Cholesterol-loaded cells were washed twice with 200 μl washing buffer (phosphate buffered saline+2 mg/mL BSA), replaced by 100 μl equilibration/efflux medium (RPMI+2 mg/ml BSA) \pm LXR/retinoid X receptor agonist (50 nM RO0721957/5 nM RO0264456) and simvastatin (0.1, 1, and 10 μM) and incubated overnight. Cells were then incubated during a 6-h efflux period with 5 $\mu\text{g}/\text{ml}$ ApoA-1 (cholesterol efflux acceptor) and simvastatin (0.1, 1, and 10 μM). Following the efflux period, supernatants were filtered and cells lysed in NP40 1 %. Supernatants and cell lysates were transferred to LumaPlate-96 (Perkin Elmer) and radioactivity determined. Cholesterol efflux was expressed as percent ^3H -activity in the medium relative to total ^3H -activity (medium and cell lysate).

J774A.1 cells Cholesterol-loaded cells were washed as described for THP-1 cells and incubated overnight with each of

five different statins (atorvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin) over a broad concentration range (0.0001–10 μM) \pm 300 μM 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate in equilibration/efflux medium (DMEM+2 mg/ml BSA). Cells were then incubated during a 6-h efflux period with 10 $\mu\text{g}/\text{ml}$ ApoA-I and statin.

Aliquots of 1.5 mg/ml HDL-C (Intracel) were incubated overnight at 4 or 37 $^{\circ}\text{C}$ with 10 $\mu\text{g}/\text{ml}$ recombinant human cholesteryl ester transfer protein and 10 μM dalcetrapib. The incubated HDL, diluted to 0.5 %, provided the cholesterol efflux acceptor during the 6-h efflux period for J774A.1 cells treated as described above.

Cell Viability Cytotoxicity was evaluated using the ApoTox-Glo Triplex Assay (Promega).

Statistical Analysis

All data are expressed as mean \pm SD. Statistical analyses employed student *t* test unless otherwise stated.

Results

Expression of miR33, ABCA1 and ABCG1 in Statin-Treated Human Macrophage THP-1 Cells

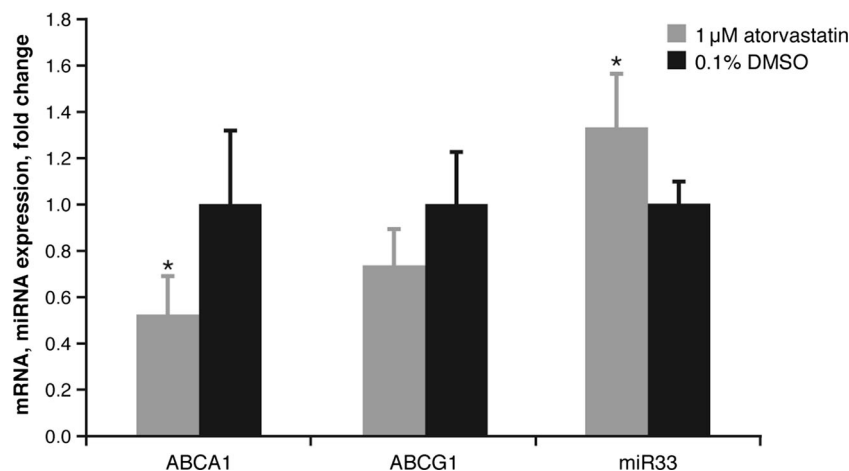
Incubation of THP-1 cells, cholesterol loaded, with 1 μM atorvastatin, was associated with a significant increase in miR33 expression (33 %, $P<0.05$) and decreased ABCA1 messenger RNA (mRNA) (–47 %, $P<0.05$) and ABCG1 mRNA (–27 %, NS) (Fig. 2). In non-cholesterol-loaded THP-1 cells, statin-mediated changes in mRNA expression of miR33, ABCA1, and ABCG1 (full data not shown, for ABCA1 and miR33 data see Fig. 3) were consistent with previous observations [21]. Simvastatin exposure of cholesterol-loaded THP-1 cells also produced a significant, dose-dependent decrease in ABCA1 mRNA, a slight increase in ABCG1 mRNA, and a significant increase in LDL receptor mRNA (Fig. 4).

ABCA1-Mediated Cholesterol Efflux in Statin Treated THP-1 Macrophages

ABCA1-mediated cholesterol efflux from cholesterol-loaded THP-1 cells to ApoA-I was reduced by a comparable degree at all tested simvastatin concentrations, and was significant for all simvastatin concentrations combined versus no simvastatin ($P<0.01$) (Fig. 4). In cholesterol-loaded J774A.1 macrophages, ABCA1 mRNA and cholesterol efflux via ABCA1 were decreased by 1 μM statin: simvastatin > pitavastatin > atorvastatin > rosuvastatin > pravastatin; i.e., the most

Fig. 2 Expression of ABCA1 and ABCG1 mRNA, and miR33, in cholesterol-loaded THP-1 cells treated with atorvastatin.

* $P < 0.05$ vs. 0.1 % DMSO. Values are mean \pm SD (triplicate). Cells incubated with acLDL for 24 h. ABC, ATP-binding cassette transporter; acLDL, acetylated LDL; DMSO, dimethyl sulfoxide; miR33, microRNA33; mRNA, messenger RNA



hydrophilic exerting the smallest effects (Fig. 5); the lowest concentration of statin tested (0.0001 μ M) did not differ from the no statin control. Following incubation of J774A.1 macrophages with simvastatin (0.0001 to 10 μ M) and 300 μ M cAMP, simvastatin significantly abrogated ABCA1-mediated cholesterol efflux, particularly at simvastatin > 0.01 μ M; efflux was virtually the same as in absence of cAMP with simvastatin ≥ 1 μ M (Fig. 6). This effect of simvastatin was associated with a virtually parallel significant increase in cytotoxicity.

We have shown previously that dalcetrapib increases the HDL remodelling activity of CETP in vitro, leading to increased pre- β 1-HDL levels, the primary acceptor of

cholesterol efflux via ABCA1 [5, 22]. When HDL was incubated with rhCETP, the addition of dalcetrapib increased ABCA1-mediated cholesterol efflux from J774A.1 macrophages (Fig. 7). However, incubation of the macrophages with simvastatin at concentrations ≥ 1 μ M virtually eliminated any HDL-ABCA1-mediated cholesterol efflux and any augmentation of that process by dalcetrapib.

Although the inhibitory effect of simvastatin on cholesterol efflux to ApoA1 is statistically significant at doses as low as 0.1 μ M in THP-1 cells not loaded with cholesterol, in the presence or absence of LXR/RXR activation, a higher concentration of simvastatin (10 μ M) is needed to measure a significant effect in cholesterol loaded THP-1 cells (Fig. 8) and only in the absence of LXR/RXR activation. Simvastatin did not affect efflux to mature HDL under any of the conditions tested.

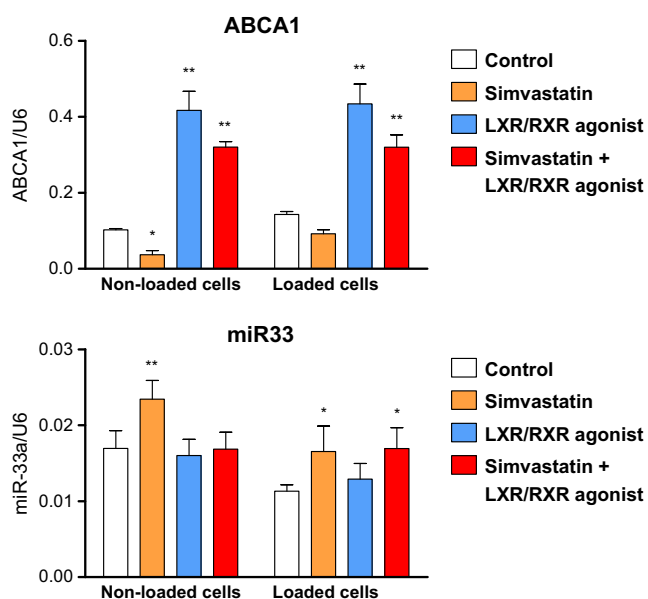


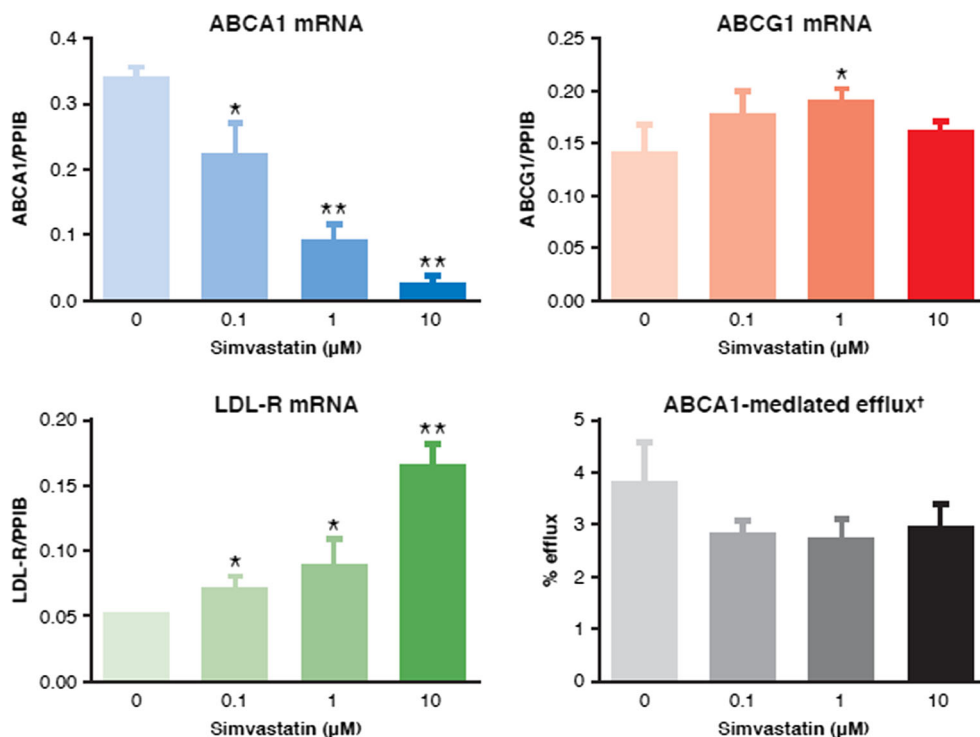
Fig. 3 Effect of simvastatin (5 μ M) treatment on miR33 expression in THP-1 cells. THP-1 cells were loaded or not with acLDL as described in the Methods section and treated with LXR/RXR agonist and simvastatin for 24 h. Data shown are mean \pm SD, one-way ANOVA, post hoc Dunnett test. * $P < 0.05$; ** $P < 0.01$ vs. control. ABC, ATP-binding cassette transporter; LXR, liver X receptor; miR33, microRNA33; RXR, retinoid X receptor

Discussion

These in vitro investigations demonstrate that statins increase miR33 expression, a suppressor of various genes including those for ABC transporters involved in RCT [20, 21]. Previous report [19] provide a putative link between statin-induced miR33 mRNA expression and reduction in both ABCA1 mRNA expression and ABCA1-mediated cholesterol efflux (Fig. 1); our results indicate that the inhibitory effect of statin (simvastatin) is specific to efflux to ApoA-I and not to mature HDL and more marked in non-cholesterol-loaded cells (Fig. 8). This is consistent with the fact that ABCA1 primarily mediates efflux to nascent HDL particles.

Our data are also consistent with previous findings that short-term treatment with low-dose atorvastatin decreased ABCA1 and ABCG1 mRNA expression in blood cells of hypercholesterolemic patients [23]. In addition, more recent studies using HDL as the lipid acceptor to evaluate ABCG1-mediated cholesterol efflux have shown that simvastatin and atorvastatin reduce cholesterol efflux in human macrophages.

Fig. 4 Effect of simvastatin treatment on ABCA1, ABCG1, LDL-R mRNA, and ABCA1-mediated cholesterol efflux in THP-1 cells. * $P < 0.05$; ** $P < 0.01$ vs. no simvastatin. † $P < 0.01$ for comparison of all simvastatin concentrations combined vs no simvastatin. Values are mean±SD (triplicate). ABC, ATP-binding cassette transporter; LDL-R, LDL receptor; mRNA, messenger RNA; PPIB, peptidyl-prolyl cis-trans isomerase B



The marked decrease in ABCA1 protein and the potential remodelling of mature HDL to lipid-poor ApoA-I, a substrate for ABCA1, may limit interpretation of the role of ABCG1 in these studies [24]. Studies with ABCA1 and ABCG1 double knockout macrophages support a predominant role of ABCA1 in the overall efflux capacity of these cells [25].

Here, we also observed that increased HDL-induced ABCA1-mediated cholesterol efflux from J774A.1 cells by dalcetrapib was abrogated by relatively hydrophobic statins (simvastatin and atorvastatin) and, to a lesser extent, by more hydrophilic statins (pravastatin and rosuvastatin), an effect not overcome by cholesterol loading of macrophages.

Horie T. et al. [26] recently demonstrated that transgenic expression of miR33b in mice decreased the expression of ABCA1 and ABCG1 reducing HDL-C by about 35 %. In

addition, macrophages from these mice showed a decreased efflux capacity to ApoA1 and HDL.

In hamsters injected with macrophages loaded with radiolabelled cholesterol we previously described an increase

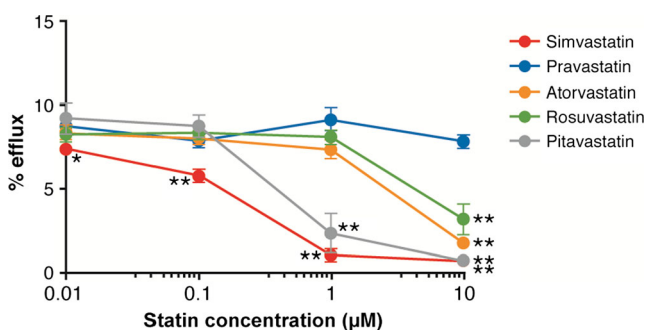


Fig. 5 Effect of different statins on ABCA1-mediated cholesterol efflux from J774A.1 macrophages. * $P < 0.05$; ** $P < 0.01$ vs. no statin. Values are mean±SD (triplicate)

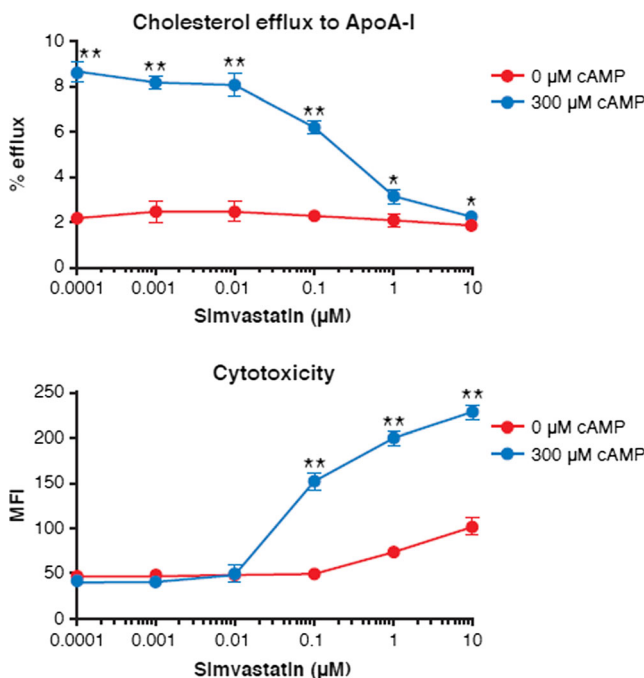


Fig. 6 Effect of ABCA1-mediated cholesterol efflux capacity on cytotoxicity of J774A.1 macrophages treated with simvastatin. * $P < 0.05$; ** $P < 0.01$ vs. no cAMP. Values are mean±SD, based on measures performed in triplicate. Apo, apolipoprotein; cAMP, 3'-5'-cyclic adenosine monophosphate; MFI, mean fluorescence index

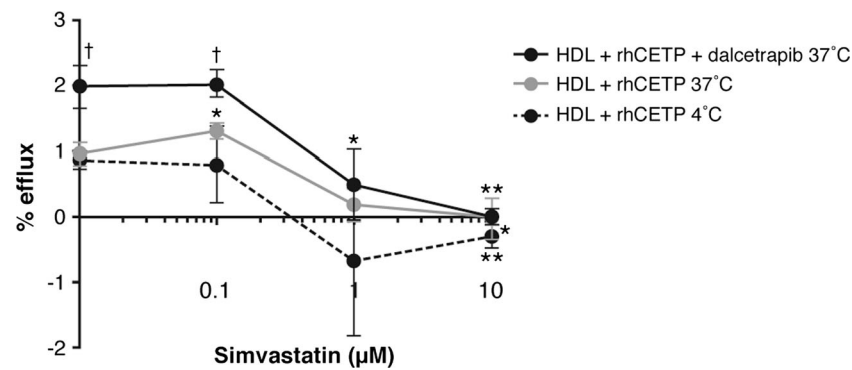


Fig. 7 Effect of dalcetrapib on ABCA1-mediated cholesterol efflux from J774A.1 macrophages. * $P < 0.05$; ** $P < 0.01$ vs. no simvastatin; † $P < 0.01$ for comparison of HDL+rhCETP 37 °C with vs. without dalcetrapib. Values are mean±SD (triplicate). Cholesterol-loaded mouse J774A.1 macrophages incubated overnight and during the 6-h efflux period with

0, 0.1, 1, and 10 µM simvastatin; HDL incubated overnight at 4 or 37 °C with 10 µg/ml CETP, and 10 µM dalcetrapib was added to the cells for the 6-h efflux period. CETP, cholesteryl ester transfer protein; rh, recombinant human

in plasma and fecal elimination of labelled sterol upon dalcetrapib treatment [5]. It should be noted that these experiments were performed in the absence of concomitant statin treatment. Recently, we reported [27] that dalcetrapib treatment of hamsters increased the intestinal absorption of the antioxidants lutein and zeaxanthin via an HDL pathway, which is ABCA1 and ApoA1 dependent. Simvastatin treatment decreased the expression of intestinal ABCA1, decreased lutein and zeaxanthin concentrations in plasma and liver, and abrogated the effect of dalcetrapib on lutein and zeaxanthin absorption. These data support the potential in vivo relevance of the in vitro observations reported here.

Interestingly, reported pro-diabetogenic effects of statins might be explained by a mechanism involving a decrease in ABCA1 expression, since ABCA1 plays a critical role in pancreatic insulin-secreting beta-cell physiology [28], while decrease in ABCA1 function may explain the observed impaired glycemic control and insulin secretion, without affecting insulin sensitivity in type 2 diabetic patients treated with statins [29]. In addition, miR33 has also been shown to modulate ABCA1 expression, cholesterol accumulation, and insulin secretion in pancreatic islets [30].

It should be noted that, although statins decrease ABCA1 expression in macrophages and intestine, these effects appear

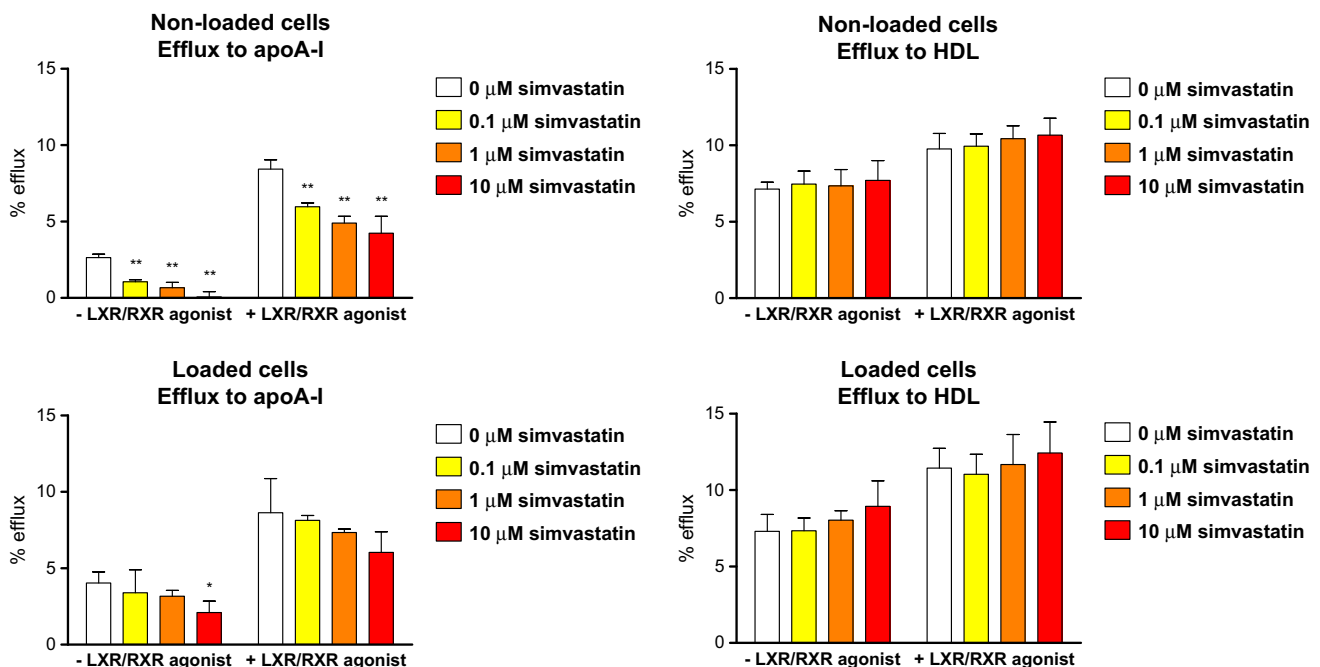


Fig. 8 Effect of simvastatin on cholesterol efflux from THP-1 cells loaded or not with cholesterol to ApoA-I vs. mature HDL particles. THP-1 cells were loaded or not with acLDL and treated with the LXR/RXR agonist and simvastatin as described in the Methods section. The

cholesterol efflux acceptors ApoA-I and HDL were used at 10 µg/ml. * $P < 0.05$; ** $P < 0.01$ vs. 0 µM simvastatin. acLDL, acetylated LDL; Apo, apolipoprotein; LXR, liver X receptor; RXR, retinoid X receptor

to be tissue-specific: statins appear to have no effect, or even to increase, expression of ABCA1 in liver (reviewed by Cerda et al. [31]). If the effect of statin treatment on HDL-C plasma level is predominantly influenced by effects on hepatic ABCA1 expression, this may explain the clinical finding that statin treatment does not reduce, and may even increase, plasma HDL-C, despite present observations indicating reduced macrophage ABCA1 expression. This hypothesis also suggests that, in statin-treated patients, the cholesterol carried by HDL may originate more from the liver than from peripheral tissues, thus explaining a less predictive and protective value of the HDL-C observed in dal-OUTCOMES.

In addition to therapeutic interventions, miR-33 expression can be upregulated in THP-1 macrophages by inflammatory conditions leading to a decrease in ABCA1 and ABCG1 expression and cholesterol efflux as very recently demonstrated by Mao et al. [32]. Thus, one may speculate that, in addition to statin treatment, the inflammatory status of Acute Coronary Syndrome patients may affect the response to HDL-raising agents.

Collectively, our observations may help reconcile the lack of clinical benefit from HDL-raising agents in statin-treated patients, with efficacy demonstrated in the absence of statin in vitro towards increasing cholesterol efflux capacity. New approaches towards HDL-raising, including a miR33 antagonist (antagomir-33), reconstituted HDL, and ApoA-I mimetics are in advanced clinical development, but to optimize their use it will be important to characterize any pharmacodynamic interaction with statin-triggered feedback on the SREBP pathways.

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