Influence of Polymorphisms and Cholesterol-Lowering Treatment on \textit{SCARB1} mRNA Expression

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\textbf{Aim}: This study evaluated the influence of polymorphisms and cholesterol-lowering treatments on \textit{SCARB1} mRNA expression in peripheral blood mononuclear cells and in HepG2 and Caco-2 cells.

\textbf{Methods}: Blood samples were drawn from normolipidemic (NL, \(n\) = 166) and hypercholesterolemic (HC, \(n\) = 123) individuals to extract DNA and total RNA and to analyze the lipid profile. After a 4-week washout period, 98 HC individuals were treated with atorvastatin (10 mg/day/4 weeks) whereas 25 were treated with ezetimibe (10 mg/day/4 weeks), followed by simvastatin (10 mg/day/8 weeks) and simvastatin plus ezetimibe (10 mg each/day/4 weeks). HepG2 and Caco-2 cells were treated with atorvastatin, simvastatin and ezetimibe at various concentrations for 12 and 24 h and collected for RNA extraction. \textit{SCARB1} mRNA expression was measured by TaqMan® assay and \textit{SCARB1} c.4G\(\rightarrow\)A, c.726+54C\(\rightarrow\)T and c.1080C\(\rightarrow\)T polymorphisms were detected by PCR-RFLP.

\textbf{Results}: High LDL cholesterol (>160 mg/dL) values were associated with low baseline \textit{SCARB1} mRNA expression in PBMC. Allele T carriers for \textit{SCARB1} c.726+54C\(\rightarrow\)T had lower basal \textit{SCARB1} transcription in PBMC \((p<0.05)\). Simvastatin, atorvastatin and ezetimibe treatments did not modify the \textit{SCARB1} mRNA level in PBMC from HC patients. Similarly, these cholesterol-lowering drugs did not modulate the \textit{SCARB1} expression in HepG2 and Caco-2 cells in spite of the concentration and time of exposure \((p>0.05)\).

\textbf{Conclusion}: LDL cholesterol levels and \textit{SCARB1} c.726+54C\(\rightarrow\)T are associated with low mRNA expression in mononuclear cells. Cholesterol-lowering drugs do not modulate \textit{SCARB1} expression in PBMC from HC subjects or in HepG2 and Caco-2 cells.


\textbf{Key words}: Scavenger receptor class B type I, Cholesterol-lowering drugs, Gene expression, Single nucleotide polymorphisms

\section*{Introduction}

The scavenger receptor class B type I (SR-BI) is a membrane protein with an important role in cholesterol traffic. SR-BI has a well-established role in the selective uptake of mature high density lipoprotein (HDL) particles and its contribution in the efflux of cholesterol from the cells to nascent HDL particles\textsuperscript{1,2}. SR-BI also participates in the uptake of apolipoprotein (apo) B-containing lipoproteins, including low density lipoprotein (LDL)\textsuperscript{3} and very low density lipoprotein (VLDL)\textsuperscript{4}. Studies using animal models have demonstrated that SR-BI has an atheroprotective role. SR-BI overexpression in the liver is associated with reduction of atherosclerosis in LDL receptor-deficient mice under a high fat and cholesterol diet\textsuperscript{5}. On the other hand, SR-BI deficiency results in lipid deposition in the aorta and atherosclerosis in mice\textsuperscript{6}. In contrast, much
less is known about the role of SR-BI in humans.

The gene encoding SR-BI (SCARB1) is composed of 13 exons located on chromosome 12q24.31-32. Alternative splicing of SCARB1 generates the variant SR-BII, in which exon 12 is omitted. SCARB1 single nucleotide polymorphisms (SNPs), and in particular three common variants at exon 1 (c.4G>A), intron 5 (c.726+54C>T) and exon 8 (c.1050C>T), have been related to impaired serum lipid levels in several populations. Moreover, these SNPs have been also associated with altered post-prandial lipid response and pharmacological interventions.

Liu and co-workers described that subjects carrying the rare allele for the SCARB1 c.4G>A SNP had higher responsiveness to fenofibrate in lowering triglycerides. We recently reported that hypercholesterolemic Brazilians carrying the TT genotype for SCARB1 c.1050C>T SNP had better LDL cholesterol response to atorvastatin, an inhibitor of cholesterol synthesis, than non-TT carriers. On the other hand, SR-BI and the Niemann-Pick C1-like 1 protein (NPC1L1) have been shown to be involved in absorption of cholesterol from the diet, which suggests that SCARB1 may be a candidate gene to modulate the response to ezetimibe, a NPC1L1 inhibitor.

Although some variants may link SCARB1 gene function with an important role in cholesterol metabolism and response to lipid-lowering drugs, there is limited information on their effects on human SCARB1 mRNA expression and its relationship with lipid metabolism disorders or its role on cholesterol-lowering therapy.

Considering the previous findings, we hypothesized that dyslipidemia, SCARB1 polymorphisms and cholesterol-lowering treatment might alter SCARB1 mRNA expression in humans. Therefore, the aim of this study was to evaluate the influence of hypercholesterolemia and three common SCARB1 SNPs (c.4G>A, c.726+54C>T and c.1050C>T) on mRNA expression using as a model peripheral blood mononuclear cells (PBMC) from normolipidemic (NL) and hypercholesterolemic (HC) Brazilian subjects. Moreover, we analyzed the influence of inhibitors of cholesterol synthesis and absorption on SCARB1 mRNA expression in PBMC from HC individuals, and the hepatic and intestinal human cell lines, HepG2 and Caco-2.

Materials and Methods

Subjects and Study Protocol

Hypercholesterolemic patients (HC, n = 123) with LDL cholesterol >160 mg/dL (4.14 mmol/L) and normolipidemic individuals (NL, n = 166) [LDL cholesterol <130 mg/dL (3.36 mmol/L); triglycerides <150 mg/dL (1.94 mmol/L)] were randomly selected at the University Hospital of the University of Sao Paulo (Sao Paulo City, SP, Brazil). Individuals with diabetes mellitus, hypertriglyceridemia, liver, renal or thyroid disease, pregnant women or those under treatment with oral contraceptives or with other causes of secondary dyslipidemia were not included in the study.

Information on age, height, weight, hypertension, obesity, tobacco smoking, alcohol consumption, physical activity, menopause status, familial history of coronary artery disease (CAD) and medication was recorded, as previously described. Each individual declared his ethnic group during the interview and agreed to participate in the study by signing an informed consent from. The study protocol was approved by the Ethics Committees of the University Hospital and School of Pharmaceutical Sciences of the University of Sao Paulo.

After a four-week washout period including a low-fat diet, HC individuals were treated with atorvastatin (ATORVA, n = 98) or simvastatin and ezetimibe (SIMVA/EZE, n = 25). The ATORVA group took 10 mg atorvastatin daily for four weeks. The SIMVA/EZE group was initially treated with ezetimibe (10 mg/day/4 weeks), followed by simvastatin (10 mg/day/8 weeks) and simvastatin plus ezetimibe (10 mg each/day/4 weeks).

Serum lipids and SCARB1 mRNA in PBMC were measured at the baseline and after each treatment. Serum alanine aminotransferase (ALT) and creatine kinase (CK) were determined for evaluation of the liver and muscular adverse reactions to cholesterol-lowering drugs.

Biochemical Measurements

Blood samples were collected after an overnight (12 h) fast. Serum total cholesterol, HDL cholesterol and triglycerides were measured by routine enzymatic colorimetric methods. Plasma apoAI and apo B were measured by nephelometry. LDL and VLDL cholesterol were estimated by the Friedewald formula. Serum ALT and CK concentrations were determined by kinetic methods to evaluate drug effects on liver and muscle tissues.

SCARB1 Genotyping

Genomic DNA was extracted from EDTA-anticoagulated blood samples using a salting-out procedure. SCARB1 SNPs c.4G>A (rs4238001), c.726+54C>T (without specified rs in the NCBI) and c.1050C>T (rs5888) (GenBank accession number NM_005505) were identified by enzymatic restriction of
PCR products (RFLP), as previously described\textsuperscript{15}.

Isolation of Mononuclear Cells from NL and HC Individuals

EDTA-anticoagulated blood was diluted in phosphate-buffered saline (1:1) and this suspension was layered in Hystopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 30 min at 400 x g at room temperature. PBMC were collected from the interphase and immediately used for RNA extraction.

Cell Culture and Treatment with Statins

Human colorectal adenocarcinoma (Caco-2) and hepatocellular carcinoma type G2 (HepG2) cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 44 mmol/L sodium bicarbonate, 10,000 U/mL streptomycin and 10,000 U/mL penicillin. Cells were grown at 37°C in a humidified atmosphere, containing 5% CO\textsubscript{2}. Culture medium was replaced twice a week and cells were trypsinized and sub-cultured every seven days.

Cells were treated with atorvastatin (kindly provided by Pfizer Pharmaceuticals Ltd., Guarulhos, SP, Brazil), simvastatin (Sigma) or ezetimibe (kindly provided by Merck/Sheering-Plough, NJ, USA). Atorvastatin was dissolved in methanol whereas simvastatin and ezetimibe were dissolved in ethanol. Simvastatin was activated by incubation with 0.1N NaOH solution at 50°C for 2 h, followed by neutralization at pH 7.0 and adjustment of the concentration to 5.6 mM. The final concentration of methanol or ethanol in the culture medium did not exceed 0.1% and 0.2%, respectively. Previous experiments showed that these concentrations of organic solvents were not cytotoxic on HepG2 and Caco-2 cells\textsuperscript{20, 21}.

Toxicity of the cholesterol-lowering drugs was evaluated by measuring the percentage of cells with loss of membrane integrity and DNA fragmentation. Cells (numbering 10,000) were analyzed in a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using an argon-ion laser (15 mW) with an incident beam at 488 nm. Red (propidium iodide) fluorescence was evaluated using a 585 nm filter. Data were acquired and analyzed using FACS/Cell Quest software (Becton Dickinson). Briefly, 24 h treatment of HepG2 cells with atorvastatin at different concentrations (0-10.0 \( \mu \text{M} \)) did not affect the membrane integrity of the cells, nor did it increase DNA fragmentation. On the other hand, Caco-2 cells treated for 24 h with atorvastatin presented with loss of membrane integrity in 15% of the cells at 10.0 \( \mu \text{M} \). For simvastatin acid, none of the concentrations tested in HepG2 (0-10.0 \( \mu \text{M} \)) or Caco-2 (0-1.0 \( \mu \text{M} \)) cells induced loss of membrane viability or DNA fragmentation after 24 h treatment. Based on these results, Caco-2 cells were treated with atorvastatin or simvastatin at 0-1.0 \( \mu \text{M} \), whereas HepG2 cells were treated with 0-10.0 \( \mu \text{M} \) up to 24 h. Finally, ezetimide did not show toxic effects at the different concentrations tested (0-5.0 \( \mu \text{M} \)) in both HepG2 and Caco-2 cells.

SCARB1 mRNA expression was measured in Caco-2 and HepG2 cells. Caco-2 cells (1.0 \( \times \) 10\textsuperscript{6} cells/75 cm\textsuperscript{2}) were cultured for three days and treated with atorvastatin, simvastatin or ezetimibe for 12 or 24 h. HepG2 cells (2.5 \( \times \) 10\textsuperscript{6} cells/75 cm\textsuperscript{2}) were cultured for 24 h and treated with atorvastatin, simvastatin or ezetimibe for 12 or 24 h.

SCARB1 mRNA Quantification by Real-Time PCR

Total RNA was extracted from PMBC, HepG2 and Caco-2 cells (1 \( \times \) 10\textsuperscript{6} to 5 \( \times \) 10\textsuperscript{6} using TRizol\textsuperscript{\textregistered} (Invitrogen-Life Technologies, CA, USA) following the manufacturer’s suggested protocol. RNA was dissolved in DEPC-treated water and the concentration was measured by spectrophotometry using NanoDrop\textsuperscript{\textregistered} (NanoDrop Technologies Inc., DE, USA). RNA integrity was evaluated using Bioanalyzer\textsuperscript{\textregistered} 2100 (Agilent Technologies, CA, USA). Samples with an RNA integrity number (RIN) lower than 5 were not used for mRNA experiments. cDNA was produced from 1 \( \mu \text{g} \) total RNA with Superscript\textsuperscript{TM} II Reverse Transcriptase (Invitrogen-Life Technologies).

SCARB1 mRNA expression was measured by quantitative TaqMan real-time PCR (qPCR). Six reference genes [ubiquitin C (UBC), glyceraldehyde-3-phosphate dehydrogenase (GAPD), beta-2-microglobulin (B2M), hypoxanthine phosphoribosyl-transferase I (HPRTI), succinate dehydrogenase complex, subunit A (SDHA) and hydroxymethyl-bilane synthase (HMBS)] were tested and analyzed using GeNorm software [http://medgen.ugent.be/genorm]. The most stable genes under experimental conditions for PBMC, HepG2 and Caco-2 cells were UBC, GAPD and HMBS, respectively. Sequences of primers and probes are described in Supplementary Table 1. The qPCR assays were carried out in 96-well plates using a 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA).

cDNA samples were assayed in duplicate for human PBMC and in triplicate for HepG2 and Caco-2 cells. The relative quantification of SCARB1 was analyzed using a comparative Ct method\textsuperscript{22} using the formula 2\textsuperscript{\textminus(\text{ΔCt})}. For HepG2 and Caco-2, using cells treated with a vehicle as a calibrator. For PBMC, no calibrator was used, and therefore the relative quantification of SCARB1 was calculated using the formula 2\textsuperscript{\textminus(\text{ΔCt})}.\textsuperscript{25}
Statistical Analysis

Statistical analyses were performed using Prism v.5.0 for Windows (Graph Pad Software Inc., CA, USA) and SPSS v.15 for Windows (SPSS Inc., Madrid, Spain). Comparisons between NL and HC individuals’ categorical variables were evaluated using the chi-square test. Continuous variables were analyzed by t-test or paired t-test and one-way ANOVA or one-way repeated measures ANOVA and Tukey’s post-hoc test. Analyses of mRNA expression data were performed using Mann Whitney and Wilcoxon tests. Correlation analysis was carried out by Spearman’s correlation coefficient. To assess the ability of independent variables to predict low SCARB1 mRNA expression, we performed multiple logistic regression analysis using the Wald Stepwise method. For this purpose, expression data were grouped into quartiles. The values in the first quartile were considered as “low expression samples”. Data from cell experiments are presented as the mean ± SEM and differences among means were tested by two-way ANOVA and Bonferroni’s post-hoc test comparing each treatment with the control. Statistical significance was set at p < 0.05.

Results

Characteristics of the Study Groups

Demographic, clinical and laboratory data are shown in Table 1. Frequencies of a family history of CAD, hypertension, obesity and menopause status were higher in the HC group than in the NL group (p < 0.05). HC patients showed higher mean age and BMI than NL individuals (p < 0.001). HC patients had a more atherogenic basal lipid profile than NL individuals, showing higher total, LDL and VLDL cholesterol, triglycerides and apoB (p < 0.05). HDL cholesterol and apoAI were similar between the NL and HC groups (p > 0.05). Serum lipid profiles in response to cholesterol-lowering treatments are shown in Supplementary Table 2.

SCARB1 mRNA Expression in PBMC

SCARB1 mRNA expression in both NL and HC individuals is shown in Fig.1A. Although SCARB1 mRNA expression was not different between the HC and NL groups, hypercholesterolemia tended to be associated with lower mRNA expression in PBMC (p = 0.053). When the sample population was grouped according to LDL cholesterol levels, individuals with the highest concentration (>160 mg/dL) showed lower SCARB1 expression than those with the lowest

Table 1. Demographic, clinical and laboratory data of the study groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>NL (166)</th>
<th>HC (123)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>47 ± 7</td>
<td>55 ± 10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ethnicity [European/African], %</td>
<td>103/60 (63/37%)</td>
<td>84/39 (68/32%)</td>
<td>0.440</td>
</tr>
<tr>
<td>Gender [female], %</td>
<td>120/46 (72%)</td>
<td>82/41 (67%)</td>
<td>0.368</td>
</tr>
<tr>
<td>Menopause, %</td>
<td>30/77 (28%)</td>
<td>63/14 (82%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Family history of CAD, %</td>
<td>69/94 (42%)</td>
<td>65/53 (55%)</td>
<td>0.046</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>60/103 (37%)</td>
<td>66/56 (54%)</td>
<td>0.005</td>
</tr>
<tr>
<td>Obesity, %</td>
<td>26/136 (16%)</td>
<td>33/86 (28%)</td>
<td>0.026</td>
</tr>
<tr>
<td>Cigarette smoking, %</td>
<td>31/135 (19%)</td>
<td>25/98 (20%)</td>
<td>0.841</td>
</tr>
<tr>
<td>Physical activity, %</td>
<td>76/89 (46%)</td>
<td>61/61 (50%)</td>
<td>0.589</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4 ± 4.2</td>
<td>27.8 ± 4.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>173 ± 19</td>
<td>272 ± 31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>98 ± 19</td>
<td>184 ± 28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>58 ± 13</td>
<td>58 ± 13</td>
<td>0.856</td>
</tr>
<tr>
<td>VLDL cholesterol, mg/dL</td>
<td>16 ± 6</td>
<td>30 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>82 ± 28</td>
<td>150 ± 58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoAI, mg/dL</td>
<td>142 ± 27</td>
<td>140 ± 26</td>
<td>0.672</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>85 ± 23</td>
<td>138 ± 28</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Number of individuals in parentheses. Values for continuous variables are presented as the mean ± SD and compared by t-test. Categorical variables were compared by chi-square test. NL, normolipidemics; HC, hypercholesterolemics; CAD, coronary arterial disease; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; apoAI, apolipoprotein AI; apoB, apolipoprotein B.
levels (<100 mg/dL) (Fig. 1B; p = 0.031).

In order to evaluate the association of independent variables with SCARB1 mRNA expression in PBMC, we grouped the data into quartiles for both NL and ATORVA groups pooled together. Individuals in the first quartile of SCARB1 mRNA levels (Q1: $2^{-\Delta CT} < 2.12 \times 10^{-3}$) were considered low expressors. Multiple logistic regression analysis was carried out to evaluate the predictors of low SCARB1 expression. LDL and HDL cholesterol and triglycerides were introduced as categorical variables using the cut-off values described as atherogenic levels by the NCEP ATP III (LDL cholesterol > 100 mg/dL; HDL cholesterol < 40 mg/dL; triglycerides > 150 mg/dL). As shown in Table 2, LDL cholesterol (OR: 2.36, CI: 1.05-5.33; p = 0.038) and age (OR: 1.04, CI: 1.00-1.08; p = 0.037) were demonstrated to contribute to low SCARB1 mRNA expression. No association was observed among other clinical variables and low SCARB1 mRNA expression.

We also performed correlation analysis between SCARB1 mRNA expression and all other continuous variables, before and after cholesterol-lowering treatments. Basal apoB was the only variable to be correlated with basal mRNA expression ($r = -0.200; p = 0.01$) (data not shown).

**Effects of Cholesterol-Lowering Drugs on SCARB1 mRNA Expression**

Results of SCARB1 mRNA expression in PBMC from HC individuals after treatment with cholesterol-lowering drugs are shown in Fig. 2. There were no significant differences in mRNA expression after treatments, suggesting that cholesterol-lowering drugs have no effect on SCARB1 mRNA expression in PBMC.

Statins do not modify the mRNA levels of SCARB1 in spite of the concentration and time of exposure in both HepG2 and Caco-2 cells. Similarly,
ezetimibe up to 5.0 μM did not influence gene expression after 24 h treatment on both cell lines (Fig. 3).

**Effects of SCARB1 Polymorphisms on mRNA Expression**

Influence of SCARB1 c.4G>A, c.726+54C>T and c.1050C>T SNPs on the serum lipid profile was previously reported in the same study population. Briefly, A allele for c.4G>A was related to lower ApoAI levels whereas allele T carriers for c.726+54C>T showed higher LDL cholesterol and ApoB concentration.

Allele T carriers for the SNP c.726+54C>T had lower mRNA expression in NL individuals and ATORVA patients before and after atorvastatin treatment (Fig. 4). SCARB1 c.4G>A and c.1050C>T did not influence the basal and posttreatment gene expression (data not shown).

We also tested the influence of genotypes adjusted by covariates, including hypercholesterolemia and other clinical variables. SCARB1 (c.4G>A, c.726+54C>T and c.1050C>T) polymorphisms were introduced as dummy variables in multiple logistic regression analysis in order to test their relationship with baseline SCARB1 mRNA expression. SNP c.726+54C>T was associated with low mRNA expression (OR: 2.38, CI: 1.01-5.60; p=0.047) (Table 2).

The influence of SCARB1 haplotypes on mRNA expression of NL and HC individuals was also evaluated. HC individuals with haplotype G1T5C8 (1-5-8, denote allele for c.4G>A, c.726+54C>T and c.1050C>T, respectively) that carry the allele variant for c.726+54C>T showed lower basal mRNA expression than non-carriers (p=0.022) (Fig. 5). A similar result was found after treatment with atorvastatin (p=0.015).

**Discussion**

In the present study, we reported the SCARB1 mRNA status in a sample of normolipidemic and hypercholesterolemic subjects from a Brazilian population, using PBMC as a study model. High LDL cholesterol values were associated with a low expression of SCARB1 in PBMC; however, there are some characteristics of SCARB1 that might cause difficulty in the interpretation and discussion of this result. First, SR-BI has multiple functions and has a dual role in cholesterol metabolism, participating in the intake and efflux of cholesterol traffic among cells and HDL particles, as well as contributing to the capture of apoB-containing lipoproteins, LDL and VLDL; second, SCARB1 displays specific expression profiles among several tissues that may result from different mechanisms of transcription regulation; and third, there is limited information explaining the factors that modulate the SCARB1 mRNA expression in humans tissues in vivo.

Complete deficiency of hepatic SR-BI was associated with significantly increased atherosclerosis in mice under high fat-cholesterol diet consumption, whereas hepatic over-expression led to reduced atherosclerosis in murine models. Although we used PBMC as a model, our results have found a modest association of hypercholesterolemia with a low expression of SCARB1 and this is in agreement with previous evidence that supports an atheroprotective role for
SR-BI in mice. Moreover, studies using SR-BI conditional knockout mice proposed that in addition to its atheroprotective role in the liver, SR-BI may exert an antiatherogenic role in extrahepatic tissues. On the other hand, no differences on SCARB1 expression were shown in human monocyte-derived macrophages from subjects with atherosclerosis compared to healthy controls. We also analyzed the influence of different clinical characteristics on low SCARB1 mRNA expression. It was observed that age, but not other clinical variables, was associated with low values of SCARB1 mRNA. Indeed, SKH-1 aged mice had lower SCARB1 expression than young mice. Supporting our results, no differences were detected on SCARB1 mRNA expression according to the hypertension status in monocytes from newly diagnosed untreated patients; however, mRNA levels of other proteins involved in the cholesterol efflux (ABCA1 and ABCG1) were decreased compared to healthy controls.

SCARB1 polymorphisms have been associated with impaired basal serum lipids and cardiovascular risk in several populations. Three SNPs have been largely studied at exon 1, intron 5 and exon 8, but only the first results in amino acid change in the final sequence of the protein; therefore, functional explanation of the influence of SCARB1 SNPs on lipid

Fig. 3. Effects of cholesterol-lowering drugs on SCARB1 mRNA expression in HepG2 and Caco-2 cells. Values are reported as the mean ± SEM and compared by two-way ANOVA comparing different concentrations with the control (0 μM). Means were obtained from three (Caco-2 and HepG2 treated with ezetimibe) or five (HepG2 treated with statins) independent experiments and expressed as a relative value of mRNA quantification to the control (2^-ΔΔCt).

Fig. 4. Influence of SCARB1 c.726+54C>T polymorphism on mRNA expression in normolipidemic (NL) and hypercholesterolemic (HC) individuals. HC individuals were treated with atorvastatin (10 mg/day/4 weeks). Values are presented as a dispersion graph with bars indicating median values and compared by the Mann-Whitney U test (*, p < 0.05).
HC individuals were treated with atorvastatin (10 mg/day/4 weeks), 1-5-8 denotes alleles for c.4G>A, c.726+54C>T and c.1050C>T polymorphisms, respectively. Values are shown as a box plot and compared by Kruskal-Wallis test (*, p < 0.05). Individuals with double heterozygotes were excluded from analysis in order to avoid unconfirmed haplotypes and individuals carrying the G1C5T8 haplotype (carriers of genotypes c.4GG/c.726+54CC/c.1050CT and c.4GG/c.726+54CC/c.1050TT) were grouped and compared with the other haplotypes. Numbers of individuals considered for haplotype comparison for G1C5C8, G1C5T8, A1C5C8 and G1T5C8 haplotypes were 47, 63, 11 and 9, respectively in normolipidemic group; and 26, 41, 3 and 5, respectively, in hypercholesterolemic group.

metabolism remains unclear. It is possible that these polymorphisms have a regulatory role by controlling gene and protein expression or altering splicing sites of the gene. These variants may also be in linkage disequilibrium with other functional variants in the SCARB1 or other proximal locus related to lipid metabolism. In addition, variants in the SCARB1 sequence have also been associated with the postprandial response to specific diets\textsuperscript{12, 13} and pharmacological interventions with lipid-lowering drugs\textsuperscript{14, 15}. In our study, we observed that the presence of the rare allele for SCARB1 c.726+54C>T alone or carried as a haplotype contributes to low SCARB1 expression.

An earlier study performed in a subset of PBMC from previously genotyped subjects demonstrated that expression levels in cells from c.726+54 CC and c.1050 TT homozygote individuals were higher than carriers of T allele for c.726+54C>T and C allele for c.1050C>T\textsuperscript{30}; however, it was difficult for the authors to establish which of the two variants was the main determinant of this difference because of the small sample size (15 subjects) and the absence of some genotypes in these subjects. On the other hand, a recent in vitro study using transfected COS cells with wild-type or c.1050C>T variant plasmids showed that this polymorphism does not alter the RNA transcript level; nevertheless, these cells had a lower level of SR-BI protein\textsuperscript{32}. However, different regulatory mechanisms may occur depending on the cellular model studied, which could explain the discrepancy with our results. Recently, it was demonstrated that in liver tissues of young females, the SCARB1 rs838896 minor allele is associated with lower mRNA expression\textsuperscript{33}; however, the common three SNPs analyzed in our study were not evaluated in that work and no linkage was found among these variants.

Increased LDL cholesterol and apoB levels were previously observed in Brazilian subjects carrying the rare allele for SCARB1 c.726+54C>T\textsuperscript{15}. Here, we have shown that high LDL cholesterol values are associated with low SCARB1 mRNA expression and apoB levels are negatively correlated with SCARB1 expression, which may provide some explanation of the influence of this variant to contribute to a more atherogenic profile, because the SR-BI works as a lipoprotein receptor responsible for selective uptake of cholesteryl esters from LDL and a decreased expression may lead to impaired SR-BI function. This is in agreement with the reported increment in apoB-containing proteins when SR-BI is down-regulated or deleted\textsuperscript{34}.

The role of SCARB1 in response to cholesterol-lowering therapy is still unclear. SCARB1 c.4G>A SNP was associated with the variation in response to fenofibrate\textsuperscript{14}, and recently we have reported an impaired response to atorvastatin associated with the c.1050C>T variant\textsuperscript{15}. In this study, we were not able to confirm the in vivo effects of simvastatin and atorvastatin on SCARB1 expression in a sample of HC individuals from a Brazilian population. Similarly in type 2 diabetic subjects, simvastatin did not influence SCARB1 expression in PBMC\textsuperscript{35}; however, the literature on the influence of statins on SCARB1 using human in vivo models is scarce.

In vitro experiments using macrophages as a model have found that pitavastatin and atorvastatin can induce the SCARB1 mRNA expression\textsuperscript{36, 37}. These data differ from our results in PBMC from HC patients. Although the effect of statins on this cell type might vary depending on the cholesterol content, it could explain why atorvastatin treatment did not alter the SCARB1 mRNA and protein expression when
THP-1 differentiated macrophages were also treated with acetyl-LDL.\(^{38}\)

It is interesting that, although \textit{SCARB1} expression in PBMC was related to LDL cholesterol contents in our population, lipid-lowering drugs that directly affect LDL cholesterol levels did not modify the mRNA expression profile in hypercholesterolemic individuals; however, it is important to consider that only individuals with a high concentration of LDL cholesterol (\(>160\) mg/dL) received lipid-lowering treatment. No effect of atorvastatin on \textit{SCARB1} expression was reported in lipid-loaded macrophages\(^{38}\) and it is possible that extensive lipid loading of PBMC prevents the activity of lipid-lowering drugs and consequently the LDL cholesterol reduction on \textit{SCARB1} expression. On the other hand, reduction of LDL cholesterol by lipid-lowering treatments and its relationship with \textit{SCARB1} mRNA in PMBC was not reflected in our \textit{in vitro} models using HepG2 and Caco-2 cells; however, different mechanisms could regulate gene expression in these different cellular models and there is no evidence that LDL cholesterol has a role in controlling \textit{SCARB1} expression in hepatic and intestinal tissues.

\textit{SR-BI} was proposed as an important transporter protein involved in intestinal cholesterol absorption, contributing with \textit{NPC1L1}, the main protein responsible for absorption of cholesterol and other lipid nutrients from the diet.\(^{16}\) During \textit{et al.} reported down-regulation of \textit{SCARB1} mRNA expression in Caco-2 cells treated with 24 \(\mu\)M ezetimibe\(^{39}\); however, the recommended dose for treatment of hypercholesterolemic patients is 10 mg per day, representing a pharmacologically active concentration of 5 \(\mu\)M in the small intestine of a human adult. Higher doses of ezetimibe provide a minimal additional lipid-altering benefit.\(^{40}\) We did not observe any change in \textit{SCARB1} expression profile when using the physiological dose of 5 \(\mu\)M ezetimibe after 24 h treatment of HepG2 or Caco-2 cells. Another study reported a down-regulation of \textit{SCARB1} when \textit{NPC1L1} was suppressed in Caco-2 cells by RNA interference (RNAi), however, the authors described that the RNAi-mediated \textit{NPC1L1} knockdown was around 90\%\(^{41}\). These disparities in the intensity of cholesterol absorption inhibition could explain the divergence between previous findings and our results from \textit{in vivo} and \textit{in vitro} models.

Our experiments using HepG2 and Caco-2 cells showed no influence of atorvastatin or simvastatin treatment on \textit{SCARB1} expression. Current knowledge regarding the impact of statins on \textit{SCARB1} regulation in hepatic and intestinal tissues is deficient. Further studies covering the influence of statins on \textit{SCARB1} regulatory mechanisms in liver and intestinal cells are still needed. Statins down-regulate liver X receptor (LXR) and up-regulate peroxisome proliferator activated receptor (PPAR) alpha in macrophages and hepatic tissues\(^{42, 43}\); two nuclear receptors known to regulate \textit{SCARB1} expression positively.\(^{44, 45}\) It is possible that the activation by statins of these two regulatory mechanisms with opposite effects could explain the lack of effects by simvastatin and atorvastatin treatments in our experiments due to a compensatory mechanism; meanwhile, more investigation is needed in order to understand the effects of statins on hepatic and intestinal tissues.

Hepatocytes and macrophages are appropriate samples for the evaluation of cholesterol transport, and hepatocytes and enterocytes are suitable for evaluating statin and ezetimibe effects, respectively; however collecting these specimens is not very convenient in human subjects. Thus, we measured the expression of \textit{SCARB1} in PBMC because these cells are easily accessible and useful as a biomarker of the influence of lipid-lowering drugs on \textit{in vivo} peripheral tissues. Although \textit{SCARB1} expression was previously evaluated in PBMC\(^{35}\), the expression of this molecule in mononuclear cells may not directly reflect the expression level in macrophages in the vascular vessel wall.\(^{40}\) On the other hand, the findings on PBMC \textit{in vivo} were similar to our findings using \textit{in vitro} models for hepatic and intestinal tissues, HepG2 and Caco-2 cells.

In summary, although more evidence is needed to confirm our findings, our results suggest that \textit{SCARB1} has an atheroprotective role in human PBMC supported by the association of high LDL cholesterol concentration with low mRNA expression in HC patients and the association of low \textit{SCARB1} mRNA expression with \(c.726+54C>T\), which has been related with increased cardiovascular risk. Furthermore, \textit{in vivo} and \textit{in vitro} experiments support the hypothesis that cholesterol synthesis and absorption inhibitors, such as statins and ezetimibe, do not influence \textit{SCARB1} mRNA expression in PBMC, HepG2 and Caco-2 cells.

\section*{Acknowledgements}

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Chile, Mario H. Hirata and Rosario D.C. Hirata are recipients from CNPq-Brazil, and Fabiana D.V. Genvigir, Raquel de Oliveira, Maria A. Willrich, and Alice C. Rodrigues are recipients from FAPESP-Brazil.

References

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39) During A, Dawson HD, Harrison EH: Carotenoid transport is decreased and expression of the lipid transporters SR-BI, NPC1L1, and ABCA1 is downregulated in Caco-2 cells treated with ezetimibe. J Nutr, 2005; 135: 2305-2312


### Supplementary Table 1. Primer and probe sequences used for mRNA quantification by Taqman® real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer and probe sequences</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| UBC  | Forward: 5'-ATTTGGGTCGCGGTTCTTG-3'  
 | Reverse: 5'-TGCCTTGACATTCTCGAT GGT-3'  
 | Probe: VIC -TCG TCA CTT GAC AAT GC-MGB/NFQ | 133 |
| GAPD | Forward: GGAAGGTGAAGGTCGGAGTCA  
 | Reverse: CTGGAAGATGGTGATGGGATTTC  
 | Probe: VIC-TCAGCCTTGACGGTGC-MGB/NFQ | 230 |
| HMBS | Forward: GGCAATGCGGCTGCAA  
 | Reverse: GGGTACCCACGCGAATCAC  
 | Probe: VIC-CGGAAGAAAACAGCC- MGB/NFQ | 64 |
| SCARB1 | Assay ID: Hs00194092_m1 (Applied biosystems, CA, USA) | 81 |

MGB/NFQ: Minor groove binding/Nonfluorescent quencher

### Supplementary Table 2. Serum lipids of hypercholesterolemic individuals treated with cholesterol-lowering drugs

**ATORVA** (n=98)

<table>
<thead>
<tr>
<th>Variables (mg/dL)</th>
<th>Baseline</th>
<th>Atorva</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>272 ± 33</td>
<td>195 ± 30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C</td>
<td>185 ± 29</td>
<td>115 ± 27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C</td>
<td>57 ± 13</td>
<td>55 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>30 ± 12</td>
<td>25 ± 10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>151 ± 59</td>
<td>126 ± 49</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoAI</td>
<td>139 ± 27</td>
<td>141 ± 29</td>
<td>0.209</td>
</tr>
<tr>
<td>ApoB</td>
<td>143 ± 29</td>
<td>92 ± 22</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**SIMVA/EZE** (n=25)

<table>
<thead>
<tr>
<th>Variable (mg/dL)</th>
<th>Baseline</th>
<th>Eze</th>
<th>Simva</th>
<th>Simva + Eze</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>270 ± 26^a</td>
<td>226 ± 30^b</td>
<td>205 ± 32^c</td>
<td>176 ± 33^d</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C</td>
<td>181 ± 26^a</td>
<td>137 ± 25^b</td>
<td>120 ± 29^c</td>
<td>92 ± 30^d</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C</td>
<td>60 ± 11</td>
<td>62 ± 11</td>
<td>60 ± 13</td>
<td>60 ± 13</td>
<td>0.312</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>29 ± 11^a</td>
<td>27 ± 13^a b</td>
<td>24 ± 10^b</td>
<td>24 ± 11^b</td>
<td>0.004</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>146 ± 54^a</td>
<td>136 ± 63^a b</td>
<td>122 ± 49^b</td>
<td>120 ± 56^b</td>
<td>0.004</td>
</tr>
<tr>
<td>ApoAI</td>
<td>147 ± 24</td>
<td>147 ± 21</td>
<td>146 ± 28</td>
<td>150 ± 21</td>
<td>0.597</td>
</tr>
<tr>
<td>ApoB</td>
<td>120 ± 19^a</td>
<td>97 ± 20^b</td>
<td>87 ± 18^c</td>
<td>74 ± 20^d</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± SD and were compared by paired t test for ATORVA group and repeated measures ANOVA for SIMVA/EZE group. a,b,c,d, different letters in the same line denote statistically significant difference. n, number of individuals; ATORVA, atorvastatin; SIMVA, simvastatin; EZE, ezetimibe; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; VLDL-C, very-low-density lipoprotein cholesterol; ApoAI, apolipoprotein AI; ApoB, apolipoprotein B.