Chapitre 17 L'ézétimibe augmente l'expression intestinale du gène du récepteur LDL chez des hommes dyslipidémiques et insulino-résistants

Jean-Philippe Drouin-Chartier, André J. Tremblay, Valéry Lemelin, Marie-Claude Lépine, Benoît Lamarche, Patrick Couture

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Résumé

Objectif : Mieux comprendre l'homéostasie intestinale du cholestérol chez des hommes avec une dyslipoprotéinémie associée à la RI en examinant l'impact de l'inhibition de l'absorption intestinale du cholestérol par l'ezetimibe sur l'expression des gènes clés impliqués dans la synthèse du cholestérol et dans la captation des lipoprotéines par le R-LDL.

Méthodes : Un total de 25 hommes avec une dyslipidémie associée à la RI ont été recrutés pour participer à cette étude randomisée, en chassé-croisé, à double insu. Les participants devaient consommer 10 mg/jour d'ezetimibe ou d'un placebo durant des périodes de 12 semaines. L'expression génique intestinale était mesurée par *PCR* quantitatif dans les biopsies duodénales collectées par gastroduodénoscopie à la fin de chaque traitement.

Résultats : Un total de 20 participants ont complété le protocole. Le traitement avec l'ezetimibe a augmenté significativement l'expression intestinale des gènes *R-LDL* (+16,2% ; *P*=0,01), *HMG-CoAR* (+14,0% ; *P*=0,04) et *ACAT-2* (+12,5% ; *P*=0,03). Les changements dans l'expression intestinale du gène *SREBP2* étaient corrélés avec les changements dans l'expression de *HMG-CoAR* (r=0,55 ; *P*<0,05), *ACAT-2* (r=0,69 ; *P*<0,001) et *PCSK9* (r=0,45 ; *P*<0,05).

Conclusions : Ces résultats montrent que l'inhibition de l'absorption intestinale du cholestérol par l'ezetimibe augmente l'expression du gène *R-LDL*. Cette étude supporte le concept voulant que l'augmentation de la clairance des LDL induite par l'ezetimibe ne se produit pas qu'au foie, mais aussi dans l'intestin.

Title page

Ezetimibe increases intestinal expression of the LDL receptor gene in dyslipidemic men with insulin resistance

Jean-Philippe Drouin-Chartier[1], André J. Tremblay[1], Valéry Lemelin[2], Marie-Claude Lépine[1], Benoît Lamarche[1], Patrick Couture[1,3]

Affiliations

1- Department of Medicine, Institute of Nutrition and Functional Foods, Laval University, Quebec City, Canada
2- Department of Gastroenterology, CHU de Québec-Université Laval, Quebec City, Canada
3- Department of Medicine, Lipid Research Center, CHU de Québec-Université Laval, Quebec City, Canada

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Ezetimibe and intestinal lipid metabolism

Address for correspondence

Patrick Couture, MD, FRCP(C), PhD
Institute of Nutrition and Functional Foods (INAF)
Laval University
2440 Hochelaga Blvd
Quebec City, QC, Canada
G1V 0A6
Phone: 418-654-2106
E-mail: patrick.couture@crchul.ulaval.ca

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Abstract

Aim: To gain further insight into intestinal cholesterol homeostasis in dyslipidaemic men with insulin resistance (IR) by examining the impact of treatment with ezetimibe on the expression of key genes involved in cholesterol synthesis and LDL receptor (R)-mediated uptake of lipoproteins.

Methods: A total of 25 men with dyslipidaemia and IR were recruited to participate in this double-blind, randomized, crossover, placebo-controlled trial. Participants received 10 mg/day ezetimibe or placebo for periods of 12 weeks each. Intestinal gene expression was measured by quantitative PCR in duodenal biopsy samples collected by gastroduodenoscopy at the end of each treatment.

Results: A total of 20 participants completed the protocol. Treatment with ezetimibe significantly increased intestinal LDLR (+16.2%; *P*=.01), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoAR; +14.0%; *P*=.04) and acetyl-Coenzyme A acetyltransferase 2 (ACAT-2) mRNA expression (+12.5%; *P*=.03). Changes in sterol regulatory element-binding transcription factor 2 (SREBP-2) expression were significantly correlated with changes in HMG-CoAR (r=0.55; *P*<0.05), ACAT-2 (r=0.69; *P*<0.001) and roprotein convertase substilisin/kexin type 9 (PCSK9) expression (r=0.45; *P*<0.05).

Conclusions: These results show that inhibition of intestinal cholesterol absorption by ezetimibe increases expression of the LDL receptor gene, supporting the concept that increased LDL clearance with ezetimibe treatment occurs not only in the liver but also in the small intestine.

Keywords: cholesterol absorption, ezetimibe, LDL receptor

Introduction

Insulin resistance is a plurimetabolic disorder caused by decreased ability of cells to uptake glucose from the bloodstream.[1] The fundamental features of insulin resistance (IR), including low HDL cholesterol levels, high TG levels, impaired glucose tolerance, high insulin levels, arterial hypertension and abdominal obesity, are all recognized as key risk factors for cardiovascular disease.[2] Our group recently showed that patients with IR present significant alterations in the expression of key intestinal genes involved in lipoprotein metabolism.[3] The small intestine is highly involved in lipoprotein and cholesterol metabolism.[4,5] Intra-enterocyte cholesterol is modulated by free cholesterol absorption from the intestinal lumen, by *de novo*-synthesized cholesterol and by uptake of circulating cholesterol-rich lipoproteins. Sterol regulatory element-binding transcription factor 2 (SREBP-2) plays an important role in this process by transcriptionally regulating key genes that are involved in intracellular cholesterol homeostasis, namely, the low density-lipoprotein receptor (LDLR), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoAR), proprotein convertase substilisin/kexin type 9 (PCSK9), Niemann-Pick C1-Like 1 (NPC1L1) and ATP-binding cassette (ABC) G5 and G8.[6]

Ezetimibe inhibits intestinal cholesterol absorption by binding to NPC1L1, decreasing LDL-cholesterol levels by 12-14%.[7-9] Ezetimibe reduces cholesterol absorption in subjects with IR,[10] which has been shown to be more important in these subjects than in insulin-sensitive subjects.[11] Ezetimibe reduces intestinal apoB-48 secretion and improves postprandial triglyceride and apoB-48 concentrations in subjects with IR.[12] These data suggest that ezetimibe has a substantial impact on intestinal lipid metabolism in patients with IR, in addition to its impact on cholesterol trafficking.

The general objective of the present study was to gain further insight into intestinal cholesterol homeostasis in dyslipidaemic men with IR, by examining the impact of treatment with ezetimibe on the expression of key genes involved in cholesterol synthesis and LDLR-mediated uptake of lipoproteins. We hypothesized that treatment with ezetimibe in dyslipidaemic men with IR significantly increases expression of the LDLR, supporting the concept that increased LDL clearance with ezetimibe treatment occurs not only in the liver but also in the small intestine.

Materials and methods

Study subjects

A total of 25 unrelated dyslipidaemic men with IR who were from the Quebec City area were recruited between December 2013 and January 2015 by the Institute of Nutrition and Functional Food of Laval University. To be included in the study, participants were required to be aged 18-65 years and to have fasting plasma triglyceride levels ≥1.3 mmol/L (114 mg/dL) and <7.0 mmol/L and fasting insulin levels ≥90 pmol/L. Fasting triglyceride and insulin levels were measured at 2 different screening visits. The

average triglyceride and insulin concentrations had to meet the above criteria. Participants also had to have a stable body weight for >3 months prior to screening, a body mass index (BMI) ≥25 kg/m^2 and a waist circumference ≥ 94 cm. Only men were included in the present study in order to limit the known confounding effects of the menstrual cycle and female hormones on lipid metabolism.[13,14] Exclusion criteria included smoking (>1 cigarette/day), illicit drug consumption, alcoholism, extreme dyslipidaemia (e.g., familial hypercholesterolaemia), type 2 diabetes, a history of cardiovascular disease or cancer, acute hepatic or renal disease (aspartate transaminase/alanine transaminase >1.5 x the upper limit of normal (ULN), creatinine levels >176 µmol/L, and creatine phosphokinase levels >2 x the ULN), HIV infection, uncontrolled high blood pressure (>160/110 mmHg) or any other condition that could interfere with participation in the study. Lipid-lowering medications were stopped 3 weeks prior to the first screening visit and were withheld for the duration of the study. Use of antidepressants, antihypertensive drugs or levothyroxine was allowed only if doses had been stable for >3 months prior to the first screening visit. Any other drugs, dietary supplements or natural health products were stopped prior to the study and were withheld for the duration of the study. The Laval University Medical Center ethics review committee approved the research protocol. Written consent was obtained from all participants. This trial was registered at clinicaltrials.gov (NCT01849068).

Study design

This study was a double-blind, randomized, crossover, placebo-controlled trial. At baseline (week 0), eligible participants were assigned to either the ezetimibe-placebo or the placebo-ezetimibe treatment sequence by a computer-assisted house program, based on their rank of inclusion in the study. Participants assigned to the ezetimibe-placebo sequence (n=13) first received 10 mg/day ezetimibe for 12 weeks and then received placebo treatment for 12 weeks without a washout period. Participants assigned to the placebo-ezetimibe sequence (n=12) received the two treatments in the reverse order. Participants were instructed to take 1 capsule with their morning meal and to maintain their usual physical activity. Alcohol consumption was limited to ≤1 serving/d during the study. During each treatment phase, the research coordinators (J.P.D.C. and M.C.L.) monitored study medication compliance by contacting the participants every 3 weeks (weeks 3, 6, and 9 of each treatment). Compliance with ezetimibe and placebo treatment was assessed by pill counting at the end of each treatment phase. Fasting blood samples were collected at the 2 screening visits, at baseline (week 0) and at the end of each treatment (weeks 12 and 24). Duodenal biopsy samples were obtained at the end of each treatment (weeks 12 and 24). Alcohol consumption and high-intensity physical activity were prohibited 48 hours prior to the duodenal biopsies. Participants and coordinators were blinded until the final statistical analyses were conducted.

Dietary assessment and counseling

Selected participants had to complete a validated web-based, self-administered food frequency questionnaire[15] before the beginning of the intervention. The food frequency questionnaire was

designed to evaluate dietary intake during the preceding 4 weeks. Based on the results of the food frequency questionnaire, participants received personalized advice from a registered nutritionist (J.P.D.C.) regarding consumption of a standard heart-healthy diet with reduced sugars and trans and saturated fats. Participants had a 2-week run-in period to familiarize themselves with the dietary recommendations (weeks -2 to 0). During the intervention, the dietary recommendations were reinforced every 3 weeks. food frequency questionnaires were also administered at weeks 12 and 24 to assess participant diets during each treatment phase.

Fasting plasma lipoprotein, glucose and insulin concentrations

Twelve-hour fasting venous blood samples were obtained from an antecubital vein. Serum was separated from blood cells by centrifugation at 1100 *g* (2200 rpm) for 10 minutes at 18°C. Serum cholesterol and TG concentrations were determined with a Roche/Hitachi MODULAR analyzer (Roche Diagnostics, Indianapolis, IN, USA) with proper reagents. Blood glucose levels were measured by colorimetry and insulin concentrations by electrochemiluminescence (Roche Diagnostics).

Intestinal biopsies

Biopsy samples were collected from the second portion of the duodenum during gastroduodenoscopy. Three samples (3 x 3 mm) were collected using single-use biopsy forceps and were immediately flash frozen in liquid nitrogen and stored at -80°C before RNA extraction.

Total RNA extraction, RNA quantification, and quantitative real-time PCR

The intestinal biopsy tissue samples were homogenized in 1 mL of Qiazol and were extracted using an RNeasy kit (Qiagen, Hilden, Germany). The tissue samples were also treated with an RNase-free DNase set to eliminate any contaminating DNA. Total RNA was then eluted into 100 µL of RNase-free H_2O and stored at -80°C. RNA quantification and quantitative real-time PCR were performed as described previously.[16] Sequence primers and gene descriptions are available in **Table S1**.

Sample size estimation

The power calculation was performed based on the expected difference in LDLR mRNA expression after treatment with ezetimibe vs placebo using a conservative approach. It was assumed that the standard deviation of the difference in mRNA levels between each treatment phase was as important as the mean treatment effect. Power calculations indicated that a total of 20 participants entering this study with a desired power of 80% would allow us to detect a treatment difference at a two-sided value of 5% if the true difference between the placebo and ezetimibe treatments was 0.935 times the within-patient standard deviation. Therefore, 25 participants were selected and randomized to account for a 20% drop-out rate.

Statistical analyses

Statistical analyses were performed using mixed models with SAS software, v9.3.0 (SAS Institute, Cary, NC, USA). In the mixed models, treatments and sequences were treated as fixed effects. Participants were treated as a random effect. mRNA gene expression and lipid risk factors were considered the dependent variables, and the treatment (ezetimibe vs placebo) was considered the independent variable in the mixed models. The covariance structure was adjusted for each dependent variable to maximize the fit of the model to the data. Treatment sequence and specific screening values (when available) were included in all models as covariables. Specific screening values were used in the models in place of treatment-specific baseline values because of the absence of a washout period between the two treatments. Statistical models for mRNA gene expression and lipid levels were adjusted for anthropometric characteristics and dietary factors that differed significantly between the two treatments. Only significant covariables were retained in the final mixed models. The normality of the models was assessed by the distribution of the scaled residual values. Because of the number of patients (n<30), a Wilcoxon non-parametric signed-rank test was also performed on the main outcomes. As part of an exploratory approach, a non-parametric Spearman's rank correlation test was performed to evaluate the associations among key intestinal genes involved in cholesterol metabolism with respect to changes in mRNA expression levels after treatment with ezetimibe vs placebo. *P* values <.05 were taken to indicate statistical significance.

Results

Characteristics of the subjects

A total of 74 men were screened, of whom 25 met the inclusion criteria and were randomized. During the intervention, five participants withdrew from the study for personal reasons (lack of time, n = 1; alcohol consumption limit considered too strict, n = 2; and no longer willing to undergo gastroduodenoscopy, n = 2). In all, 20 men completed the study. Participant demographic, anthropometric and fasting biochemical characteristics are presented in **Table 1**. The mean age, BMI and waist circumference of the participants were 39.4 ± 10.8 y, 34.4 ± 3.9 kg/m^2 and 113.9 ± 10.8 cm, respectively. Participants with IR presented with an elevated fasting insulin level of 138 ± 30 pmol/L and a homeostasis model assessment of insulin resistance (HOMA-IR) index of 4.63 ± 0.95. Subjects also presented with the typical findings of atherogenic dyslipidaemia, including increased plasma triglyceride levels (2.25 ± 0.67 mmol/L) and low HDL cholesterol levels (1.01 ± 0.24 mmol/L).

During the intervention, compliance with treatment was very high and was similar between the two phases (placebo: 97.2 ± 4.6%; ezetimibe: 96.7 ± 4.0%; *P* = .7). All participants had compliance scores >80% during each phase.

Dietary intake was similar between the two treatment phases, with the exception of lipid and saturated fatty acid (SFA) intake (**Table 2**). Lipid intake was slightly but significantly higher during treatment with ezetimibe (Δ = +1.6% of calories; *P* = .04), which was due to an increase in SFA intake (Δ = +0.9% of calories; *P* = .03).

Table 3 shows the anthropometric and biochemical characteristics of the participants at the end of each treatment. LDL cholesterol levels were significantly reduced after treatment with ezetimibe compared to placebo (Δ = -23.3%; *P* < .0001). As shown in **Figure 1**, LDL cholesterol levels decreased in 19 of 20 participants. Total cholesterol levels and the total cholesterol/HDL cholesterol ratio also decreased after treatment with ezetimibe (Δ = -14.5% and Δ = -15.8%, *P* < .0001, respectively), while ezetimibe had no significant impact on HDL cholesterol levels. Twelve of 20 participants had lower triglyceride levels with ezetimibe treatment, but the mean reduction did not reach statistical significance (Δ = -11.9%; *P* = .09). Fasting glucose levels were significantly higher following treatment with ezetimibe vs placebo (Δ = +2.9%; *P* = .03), but insulin levels and the HOMA-IR remained unchanged.

Intestinal mRNA expression

Table 4 presents the changes in intestinal mRNA expression of various genes involved in cholesterol and lipoprotein metabolism. Treatment with ezetimibe significantly upregulated intestinal expression of the LDLR (Δ = +16.2%; *P* = .01); however, the expression of PCSK9 and other genes involved in lipoprotein assembly and transport did not change after ezetimibe therapy. Of the genes involved in cholesterol metabolism and transport, HMG-CoAR (Δ = +14.0%; *P* = .04) and acetyl-Coenzyme A acetyltransferase 2 (ACAT-2) (Δ = +12.5%; *P* = .03) were significantly upregulated by ezetimibe. Intestinal NPC1L1, ABCG5 and ABCG8 mRNA levels were not changed. Ezetimibe had no impact on the intestinal mRNA expression of genes that are involved in fatty acid metabolism and transport, such as acyl-CoA synthetase 1 (ACS-1), fatty acid transport protein 4 (FATP-4), and fatty acid binding protein 2 (FABP-2). No significant changes were observed in the expression of genes involved in triglyceride synthesis, but a slight down-regulation in mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase (MGAT-2) expression (Δ = -5.8%; *P* = .06) was noted. This decrease reached statistical significance only in the non-parametric analysis (p value for Wilcoxon signed-rank test = .03). Hepatocyte nuclear factor-4α (HNF-4α) mRNA levels were significantly reduced by ezetimibe (Δ = -9.6%; *P* = .007).

Figure 2 shows the individual changes in mRNA levels in response to ezetimibe for the LDLR, HMG-CoAR, ACAT-2 and HNF-4α. Intestinal LDLR, HMG-CoAR and ACAT-2 mRNA expression increased in 14 of 20 participants. Regarding HNF-4α, intestinal mRNA expression decreased in 15 participants.

Table 5 shows the correlations among key intestinal genes involved in cholesterol and lipoprotein metabolism with respect to changes in intestinal mRNA expression induced by ezetimibe. Changes in SREBP-2 expression were significantly correlated with changes in HMG-CoAR (r = 0.55; *P* < .05), ACAT-2 (r = 0.69; *P* < .001), ACS-1 (r = 0.70; *P* < .001) and PCSK9 expression (r = 0.45; *P* < .05). Similarly, changes in HNF-4α expression were correlated with changes in NPC1L1 (r = 0.62; *P* < .01), ABCG5 (r = 0.56; *P* < .05) and ABCG8 expression (r = 0.51; *P* < .05). Changes in ABCG5 expression were highly correlated with changes in ABCG8 expression (r = 0.91; *P* < .001). Finally, no correlations were observed between changes in gene expression and changes in lipid levels.

Discussion

In the present study we evaluated the impact of 12 weeks of treatment with ezetimibe (10 mg/day) on intestinal cholesterol homeostasis in dyslipidaemic men with IR. Treatment with ezetimibe was associated with a 23% reduction in plasma LDL cholesterol levels. In addition, ezetimibe significantly increased the levels of intestinal LDLR (+16.2%), HMG-CoAR (+14.0%), and ACAT-2 (+12.5%) mRNA expression and reduced the mRNA expression level of HNF-4α (-9.6%). Our study showed, for the first time that ezetimibe regulates several key genes involved in intestinal lipoprotein metabolism in humans.

In humans, NPC1L1 is expressed in the intestine on the apical surface of enterocytes[17] and in the liver on the canalicular membrane of hepatocytes.[18] In the intestine, NPC1L1 mediates free cholesterol absorption, which occurs mainly in the duodenum and jejunum.[17] Some evidence suggests that hepatic NPC1L1 prevents excessive cholesterol loss in the bile by counterbalancing cholesterol excretion, which is facilitated by hepatic ABCG5 and ABCG8.[18,19] By inhibiting intestinal and hepatic NPC1L1 expression, ezetimibe decreases cholesterol absorption and its delivery from the intestine to the liver. This disruption of the enterohepatic circulation induces compensatory processes that modify cholesterol homeostasis and indirectly decrease LDL cholesterol levels.[17,18,20]

The upregulation of the LDLR gene observed in the present study is in agreement with several studies conducted in animal models that reported increases in intestinal and hepatic LDLR expression with ezetimibe therapy.[21,22] The upregulation of intestinal LDLR expression at least partially explains the increase in the whole-body LDL fractional catabolic rate previously reported in patients treated with ezetimibe[23, 24] and confirms that the effects of ezetimibe on LDL cholesterol levels in humans result from both enhanced cholesterol excretion and increased clearance of circulating cholesterol-rich lipoproteins. Studies in mammals have shown that the small intestine accounts for 7-15% of whole-body LDL uptake.[5, 25] Our study suggests that the small intestine may play a role in LDL clearance and whole-body cholesterol homeostasis in humans.

PCSK9 binds to the LDLR and directs it toward lysosomal degradation rather than normal recycling in the cell membrane.[26] In human HepG2 cells, ezetimibe has been shown to enhance PCSK9 mRNA expression without affecting PCSK9 protein secretion.[27,28] Several clinical trials have also reported that ezetimibe has no significant impact on circulating PCSK9 levels.[29-31] In contrast, Engelking et al.[22] reported that ezetimibe increases jejunal mRNA expression of the LDLR (~2-fold), PCSK9 (~3-fold) and SREBP-2 (~1.3-fold) in mice. Concomitant up-regulations in intestinal LDLR, PCSK9 and SREBP-2 mRNA and protein expression were also reported in rats[28] and cynomolgus monkeys.[32] Although both LDLR and PCSK9 are known to be transcriptionally regulated by SREBP-2,[6, 33] the increase in intestinal LDLR mRNA expression observed in the present study did not correlate with a concomitant up-regulation in intestinal PCSK9 mRNA expression, which suggests that the concentration and activity of the intestinal LDLR protein were increased. Further studies are needed to confirm our findings and to elucidate the effects of ezetimibe on PCSK9 expression in humans.

Using surrogate markers of cholesterol homeostasis, Sudhop et al.[34, 35] showed that ezetimibe increases cholesterol synthesis by >70%. Similarly, our results showed a 14% increase in intestinal HMG-CoAR mRNA expression; HMG-CoAR is the rate-limiting enzyme in the endogenous cholesterol synthesis pathway. The increase in intestinal HMG-CoAR expression with ezetimibe therapy has been previously documented in animal models,[21, 22, 36] and this observation suggests that enterocytes counterbalance cholesterol depletion by increasing cholesterol synthesis, in addition to increasing cholesterol uptake from circulating lipoproteins. The increase in HMG-CoAR expression was also associated with significant upregulation of the expression of ACAT-2, which catalyzes cholesterol esterification[37] and is essential for intestinal cholesterol absorption.[38, 39] ACAT-2 upregulation may result from the increased cholesterol synthesis[37] and/or is a compensatory response to counterbalance ezetimibe and stimulate intestinal cholesterol absorption.[38, 39] In mice treated with ezetimibe, Sandoval et al.[40] measured an upregulation in ACAT-2 while Wang et al.[41] measured a down-regulation. Authors suggested that the decreased cholesterol content of enterocytes and lipid transport caused these alterations in ACAT-2 expression.[40, 41] Further investigations are clearly needed to assess the effect of ezetimibe on ACAT-2 intestinal expression in humans.

The present results indicate that treatment with ezetimibe had no impact on intestinal NPC1L1 mRNA expression, suggesting that NPC1L1 undergoes post-transcriptional regulation. Previous studies showed no significant impact of ezetimibe on NPC1L1 expression in various animal models.[22, 36] Telford et al.,[21] however, observed a significant increase in NPC1L1 mRNA expression in the jejunum of miniature pigs after ezetimibe treatment. In the present study, ezetimibe had no significant effect on intestinal SREBP-2 expression but significantly reduced HNF-4α mRNA levels; SREBP-2 and HNF-4α regulate NPC1L1 expression.[42] This finding suggests that HNF-4α has a limited impact on intestinal NPC1L1 expression in humans.

HNF-4α is a nuclear transcription factor involved in glucose, cholesterol and fatty acid metabolism. Intestinal HNF-4α has previously been shown to promote the expression of FATP-4,[43] FABP-2,[44] MTP[45] and apoA-IV.[45] The present results support these findings and, for the first time in humans, showed that changes in the expression of HNF-4α were correlated with changes in FATP-4 expression, suggesting that HNF-4α contributes to regulation of the expression of FATP-4 in the human intestine.

Intra-enterocyte cholesterol concentration is the outcome of transcriptional regulation of *de novo* cholesterol synthesis, intestinal cholesterol absorption, cholesterol efflux and cholesterol uptake from cholesterol-rich lipoproteins by SREBP-2.[6] The present study supported this concept by showing that inhibition of cholesterol absorption by ezetimibe is associated with higher intestinal LDLR expression, which most likely leads to enhanced LDL particle uptake. Interestingly, changes in SREBP-2 expression were significantly correlated with changes in HMG-CoAR, ACAT-2 and PCSK9 expression, confirming the transcriptional regulation of these genes by SREBP-2 in the human intestine; however, the relatively small sample size of this study may have limited the statistical power to detect significant changes in the expression of other key genes involved in cholesterol and fatty acid metabolism. The ABCA1-mediated cholesterol transport was not evaluated in the present study. ABCA1 plays an important role in cholesterol absorption and in HDL biosynthesis.[46-48] In mice and in intestinal Caco-2 cells, ezetimibe decreased ABCA1 intestinal expression.[49, 50] One can speculate that ABCA1 intestinal expression was also downregulated following treatment with ezetimibe in the present study, leading to reduced HDL-C secretion as a compensatory effect to maintain intra-enterocyte cholesterol content. Moreover, treatment with ezetimibe has been showed to enhance macrophage-to-faeces reverse cholesterol transport, suggesting another potential mechanism underlying cholesterol-lowering effects of ezetimibe.[51, 52] However, this remains to be thoroughly evaluated in humans.

The lipid-lowering and cardioprotective effects of ezetimibe have been shown to be more important in patients with IR than in insulin-sensitive patients,[11, 53] but the mechanisms underlying these effects remain unknown. In insulin-receptor knockout mice, PCSK9 levels were increased, and LDLR protein levels were suppressed, suggesting a major role for insulin signaling in post-transcriptional regulation of the LDLR.[54] In humans, IR has been associated with elevated PCSK9 levels, suggesting that LDLR expression is down-regulated in patients with IR compared with insulin-sensitive patients.[55] One can speculate, therefore, that the greater lipid-lowering effects of ezetimibe in IR patients compared with insulin-sensitive patients may be attributable to greater upregulations of LDLR expression levels. Further studies are needed to confirm this hypothesis.

This study had several strengths but also had some limitations that need to be outlined. To our knowledge, this was the first double-blind, randomized, crossover, placebo-controlled study to

examine the impact of ezetimibe on the intestinal expression of key genes involved in lipoprotein metabolism in humans. The study design reduced the intra-individual variability of the results and thus increased the statistical power of the study. The statistical analyses were also undertaken in a blinded fashion and were based on an *a priori* defined plan and hypothesis. The validity of the duodenal model used in this study has been demonstrated in several previous studies, making the results very robust;[3, 56, 57] however, the relatively small sample size of the study limited its statistical power to correlate changes in intestinal mRNA expression with lipid levels.

In conclusion, treatment with ezetimibe significantly increased intestinal LDLR expression, supporting the concept that increased LDL clearance with ezetimibe treatment occurs not only in the liver but also in the small intestine. Treatment with ezetimibe also significantly increased the duodenal mRNA levels of HMG-CoAR and ACAT-2, which are involved in *de novo* cholesterol synthesis.

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Author contributions

P.C. and B.L. designed the study and obtained funding. J.P.D.C., M.C.L., A.J.T. and V.L. conducted the research. J.P.D.C., A.J.T. and P.C. analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Tables

Table 17-1 Characteristics of the participants at screening (n=20)

	Mean ± s.d.
Age, years	39.4 ± 10.8
Weight, kg	106.1 ± 16.1
BMI, kg/m^2	34.4 ± 3.9
Waist circumference, cm	113.9 ± 10.8
Systolic blood pressure, mm Hg	125 ± 9
Diastolic blood pressure, mm Hg	76 ± 8
Total cholesterol, mmol/L	5.29 ± 1.03
Triglycerides, mmol/L	2.25 ± 0.67[1]
HDL cholesterol, mmol/L	1.01 ± 0.24
LDL cholesterol, mmol/L	3.30 ± 0.93
Total cholesterol/HDL cholesterol	5.37 ± 1.23
Glucose, mmol/L	5.26 ± 0.67
Insulin, pmol/L	138 ± 30[1]
HOMA-IR	4.60 ± 0.96

s.d., standard deviation;

[1]Triglycerides and insulin were measured at the two screening visits, and values are presented as the mean (± s.d.) for the two screening visits.

Table 17-2 Dietary intake during the two treatments (n=20)

	Placebo (mean ± s.d.)	**Ezetimibe (mean ± s.d.)**	**Δ (mean ± s.e.m.)**	***P1***	***P2***
Energy, kcal	2626 ± 796	2476 ± 949	-150 ± 150	.3	.2
Alcohol, %	1.7 ± 2.0	1.3 ± 1.6	-0.4 ± 0.2	.08	.2
Lipids, %	34.1 ± 3.6	35.7 ± 3.4	+1.6 ± 0.7	.04	.07
SFA, %	11.9 ± 2.0	12.8 ± 1.9	+0.9 ± 0.4	.03	.04
MUFA, %	13.6 ± 2.2	14.1 ± 1.8	+0.5 ± 0.3	.1	.1
PUFA, %	5.8 ± 0.8	5.8 ± 0.9	0.0 ± 0.1	.7	.6
TFA, %	1.6 ± 0.3	1.7 ± 0.3	+0.1 ± 0.1	.1	.1
Dietary cholesterol, mg	345 ± 157	329 ± 144	-16 ± 20	.4	.2
Protein, %	17.6 ± 2.0	17.9 ± 2.8	+0.3 ± 0.7	.7	.7
Carbohydrates, %	48.6 ± 4.9	47.1 ± 5.7	-1.5 ± 1.0	.2	.2
Fiber, g	27.5 ± 9.4	24.9 ± 10.7	-2.6 ± 1.7	.2	.08

s.d., standard deviation; s.e.m., standard error of the mean; SFA saturated fatty acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, trans fatty acids.

P1: *P*-value from mixed models. Dietary intake, which was measured at the screening and during the treatment sequences, was included as a covariable in the model. Covariables remained in the model only if their effect was significant.

P2: *P*-value from the Wilcoxon non-parametric signed-rank test.

Table 17-3 Anthropometric and biochemical characteristics of the participants at the end of each treatment (n=20)

	Placebo (mean ± s.d.)	**Ezetimibe (mean ± s.d.)**	**%Δ (mean ± s.e.m.)**	***P1***	***P2***
Weight, kg	105.9 ± 16.8	106.3 ± 17.5	+0.4 ± 0.5	.5	.4
BMI, kg/m^2	34.3 ± 4.1	34.4 ± 4.2	+0.3 ± 0.5	.6	.4
Waist circumference, cm	115.5 ± 12.1	115.5 ± 12.3	0.0 ± 0.4	.9	.9
Total cholesterol, mmol/L	5.39 ± 1.09	4.61 ± 0.77	-14.5 ± 2.4	<.0001	<.0001
Triglycerides, mmol/L	2.26 ± 0.87	1.99 ± 0.71	-11.9 ± 6.7	.09	.1
HDL cholesterol, mmol/L	1.03 ± 0.27	1.05 ± 0.24	+1.9 ± 2.2	.4	.4
LDL cholesterol, mmol/L	3.44 ± 1.06	2.64 ± 0.55	-23.3 ± 4.7	<.0001	<.0001
Total cholesterol/HDL cholesterol	5.37 ± 1.10	4.52 ± 1.06	-15.8 ± 2.4	<.0001	<.0001
Glucose, mmol/L	5.16 ± 0.38	5.31 ± 0.58	+2.9 ± 1.3	.03	.02
Insulin, pmol/L	135 ± 45	147 ± 54	+8.9 ± 6.8	.2	.2
HOMA-IR	4.41± 1.43	4.91 ± 1.61	+11.3 ± 7.0	.1	.1

s.d., standard deviation; s.e.m., standard error of the mean.

P1: *P*-value from mixed models. Pre-intervention screening values and treatment sequences were included as covariables in the model. For lipids, treatment-specific saturated fatty acid intake was also included *a priori* in the model. Covariables remained in the model only if their effect was significant.

P2: *P*-value from the Wilcoxon non-parametric signed-rank test.

Table 17-4 Intestinal expression of key genes involved in intestinal cholesterol and lipoprotein metabolism

	Placebo (mean ± s.d.)	Ezetimibe (mean ± s.d.)	%Δ (mean ± s.e.m.)	*P1*	*P2*
Nuclear transcription factors					
SREBP-1c	160,187 ± 43,207	158,979 ± 33,123	-0.8 ± 5.0	.2	.9
SREBP-2	182,349 ± 36,681	193,012 ± 32,787	+5.8 ± 4.7	.2	.3
HNF-4α	611,337 ± 123,333	552,615 ± 65,508	-9.6 ± 4.3	.007	.009
Lipoprotein assembly and transport					
LDLR	95,140 ± 31,840	110,531 ± 32,604	+16.2 ± 6.3	.01	.02
PCSK9	8,091 ± 6,131	9,079 ± 5,816	+12.2 ± 15.5	.2	.5
MTP	4,057,854 ± 1,073,496	3,799,315 ± 820,831	-6.4 ± 7.1	.4	.3
ApoB	3,657,998 ± 1,284,821	3,234,443 ± 791,734	-11.6 ± 7.2	.1	.07
ApoB-48R	15,219 ± 4,564	14,992 ± 3,266	-1.5 ± 5.8	.8	.8
ApoA-1	51,359,744 ± 25,222,842	42,796,864 ± 17,404,344	-16.7 ± 11.7	.4	.3
Cholesterol metabolism and transport					
HMG-CoAR	311,268 ± 88,237	354,911 ± 102,607	+14.0 ± 6.2	.04	.03
NPC1L1	448,686 ± 138,746	454,298 ± 97,707	+1.3 ± 7.9	.9	.9
ABCG5	283,863 ± 132,434	271,811 ± 111,688	-4.6 ± 8.9	.6	.5
ABCG8	129,551 ± 63,246	125,315 ± 46,842	-3.3 ± 9.5	.7	.7
ACAT-2	169,288 ± 45,592	190,490 ± 46,536	+12.5 ± 6.4	.03	.04
Fatty acid metabolism and transport					
ACS-1	127,371 ± 47,293	135,730 ± 40,654	+6.6 ± 7.2	.4	.3
FABP-2	762,271 ± 253,083	693,918 ± 166,298	-9.0 ± 7.1	.2	.1
FATP-4	236,181 ± 43,611	237,536 ± 31,673	+0.6 ± 6.9	.2	.06
TG synthesis					
MGAT-2	310,945 ± 47,187	293,030 ± 31,704	-5.8 ± 2.9	.06	.03
DGAT-1	1,391,347 ± 326,964	1,317,824 ± 246,771	-5.3 ± 5.8	.4	.3
DGAT-2	236,076 ± 111,334	226,428 ± 96,297	-4.1 ± 10.7	.7	.6

s.d., standard deviation; s.e.m., standard error of the mean. Data are presented as no. of copies/100,000 copies of the house keeping gene, TATA-box binding protein (TBP). ACAT2, APOA1 and PCSK9 expression values were log transformed prior to analysis in mixed models. *P1*: *P*-value from mixed models. Treatment sequence and treatment-specific saturated fatty acid intake were added *a priori* as covariables in the models. Covariables remained in the model only if their effect was significant. ACAT2, APOA1 and PCSK9 expression values were log transformed prior to analysis to enhance the normality of the models. *P2*: *P*-value from the Wilcoxon non-parametric signed-rank test.

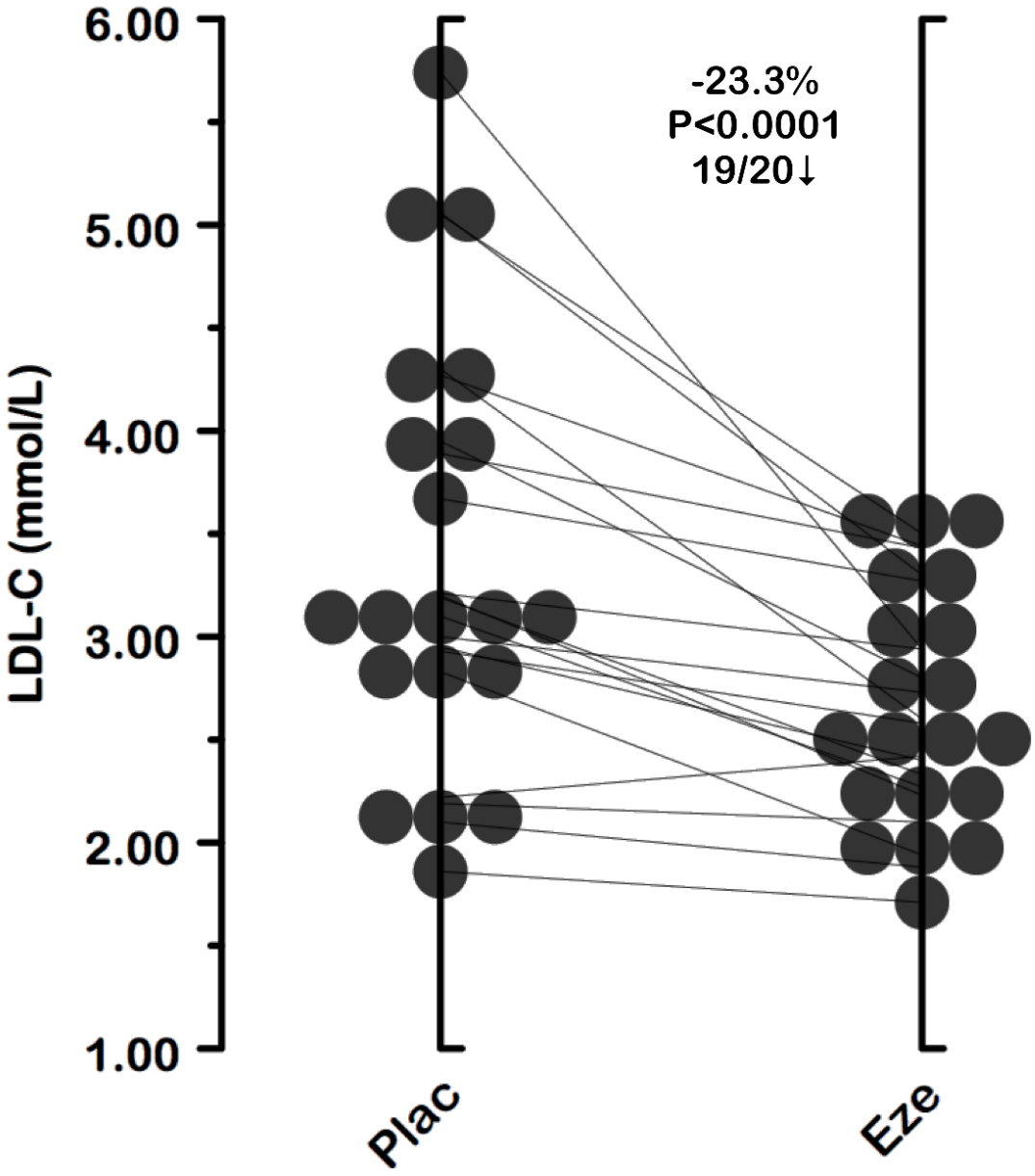
Table 17-5 Correlations among key intestinal genes involved in cholesterol metabolism with respect to changes in intestinal mRNA expression levels induced by ezetimibe

	SREBP-1c	SREBP-2	HNF-4α	HMG-CoAR	ACAT-2	NPC1L1	ABCG5	ABCG8	ACS-1	FABP-2	FATP-4	MGAT-2	DGAT-1	DGAT-2	MTP	ApoB	ApoB-48R	ApoA-1	LDLR
SREBP-2	0.43																		
HNF-4α	0.66†	0.39																	
HMG-CoAR	0.27	0.55*	0.42																
ACAT-2	0.083	0.69‡	0.34	0.85‡															
NPC1L1	0.41	0.00	0.62†	0.27	0.24														
ABCG5	0.51*	-0.13	0.56*	0.22	-0.03	0.81‡													
ABCG8	0.37	-0.25	0.51*	0.14	-0.08	0.67†	0.91‡												
ACS-1	0.25	0.70‡	0.14	0.51*	0.50*	0.08	-0.07	-0.19											
FABP-2	-0.08	-0.13	0.33	0.23	0.17	0.61†	0.55*	0.54*	0.03										
FATP-4	0.23	-0.09	0.45*	0.23	0.15	0.77‡	0.75‡	0.75‡	0.05	0.7‡									
MGAT-2	-0.12	0.24	0.34	0.60†	0.51*	0.39	0.25	0.21	0.47*	0.73‡	0.52*								
DGAT-1	-0.09	-0.13	0.26	0.08	0.08	0.65†	0.46*	0.46*	0.19	0.81‡	0.81‡	0.64†							
DGAT-2	-0.19	-0.09	0.14	0.03	-0.02	0.38	0.31	0.31	0.11	0.72‡	0.37	0.50*	0.66†						
MTP	0.07	-0.09	0.50*	0.26	0.27	0.81‡	0.64†	0.65†	-0.03	0.85‡	0.83‡	0.58†	0.81‡	0.52*					
ApoB	0.49*	-0.14	0.50*	0.11	-0.02	0.88‡	0.90‡	0.76‡	-0.04	0.50*	0.64†	0.15	0.42	0.37	0.64†				
ApoB-48R	0.21	0.19	0.40	-0.09	0.06	0.39	0.26	0.13	-0.23	0.17	0.06	-0.08	0.08	0.33	0.27	0.34			
ApoA-1	0.18	-0.05	0.54*	0.08	0.13	0.78‡	0.55*	0.42	-0.08	0.57†	0.53*	0.29	0.57†	0.58†	0.65†	0.69‡	0.58†		
LDLR	0.43	0.29	0.38	0.51*	0.37	0.26	0.31	0.39	0.34	0.15	0.40	0.25	0.25	-0.17	0.35	0.13	-0.14	-0.08	
PCSK9	-0.01	0.45*	0.31	0.83‡	0.82‡	0.22	0.05	0.00	0.30	0.29	0.12	0.67†	0.05	0.13	0.29	0.03	0.03	0.10	0.19

Correlations were measured using Spearman's rank test. *$p < .05$; †p < .01; ‡ $P < .001$.

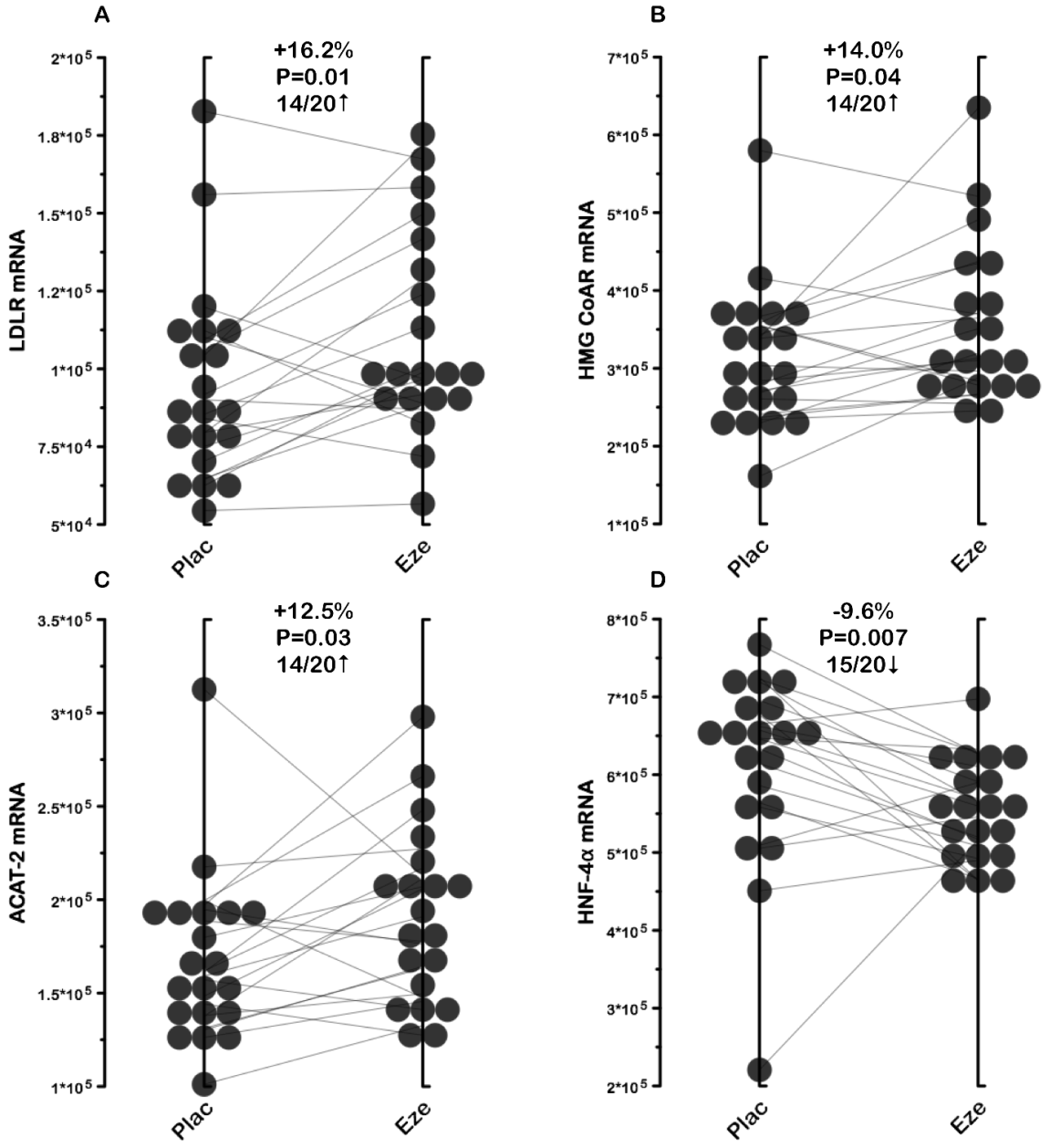
Figures

Figure 17-1 Individual changes in plasma LDL-C levels between the placebo and ezetimibe treatments



Individual changes in plasma LDL-C levels between the placebo and ezetimibe treatments (n=20). Standard deviations of the mean concentrations of both treatments are illustrated as error bars on each side of the graph.

Figure 17-2 Individual changes in intestinal A) LDLR, (B) HMG-CoAR, (C) ACAT-2 and (D) HNF-4α mRNA levels between the placebo and ezetimibe treatments



Individual changes in intestinal A) LDLR, (B) HMG-CoAR, (C) ACAT-2 and (D) HNF-4α mRNA levels between the placebo and ezetimibe treatments (n=20). Standard deviations of the mean mRNA expression levels of both treatments are illustrated as error bars on each side of the graph.

Supplemental material

Supplemental table 17-1 Sequence primers and gene descriptions

Gene Symbol	Description	GenBank	Size (bp)	Primer sequence 5'→3' S/AS
DGAT1	Homo sapiens diacylglycerol O-acyltransferase 1 (DGAT1)	NM_012079	135	TGCAGGATTCTTTATTCAGCTCT/CCACCAGGATGCCATACTTGAT
FABP2	Homo sapiens fatty acid binding protein 2, intestinal (FABP2)	NM_000134	137	TCAGGCTGGAATGTAGTGGAGAGA/CAAAACAAAAATTAGCTGGGCACTG
HMGCoAR	Homo sapiens 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), 2 transcripts	NM_000859	195	GGGACCAACCTACTACCTCAG/CGACCTGTTGTGAATCATGTGACTT
ABCG5	Homo sapiens ATP-binding cassette, sub-family G (WHITE), member 5 (ABCG5)	NM_022436	196	AGGCATGCTGAACGCTGTGAATC/TCGGGCAACCTCAGGATGTAA
ABCG8	Homo sapiens ATP-binding cassette, sub-family G (WHITE), member 8 (ABCG8)	NM_022437	269	GGGCAATGCTTTACTATGAACTGGA/ATTGCTGAAGAAGGAGGCCATGT
FATP4	Homo sapiens solute carrier family 27 (fatty acid transporter), member 4 (SLC27A4)	NM_005094	139	TGGCTGCCCTGGTGTACTATG/TTCCGAATCACCACCGTCATG
MGAT2	Homo sapiens mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase (MGAT2)	NM_002408	191	TAACCGGCCCGAATACCTCAG/AGGGTACAACTGAATGCTGAAAGGAAA
DGAT2	Homo sapiens diacylglycerol O-acyltransferase 2 (DGAT2), 2 transcripts	NM_032564	215	CCGATGGGTCCAGAAGAAGTT/TCACCAGGGCCTCCATGTACA

MTP	Homo sapiens microsomal triglyceride transfer protein (MTTP), 2 transcripts	NM_000253	210	CAGGGTGGTCTAGCTATTGATATTTC/TGGGTACTGAGAAAACTGCACTGT
APOB	Homo sapiens apolipoprotein B (APOB)	NM_000384	274	CTGCGCAACGAGATCAAGACA/CATGCTGGGAATCGACTTGTGA
LDLR	Homo sapiens low density lipoprotein receptor (LDLR)	NM_000527	193	GCCGTAAGGACACAGCACACAACC/GGAGCACGATGGGGAGGACAAT
PCSK9	Homo sapiens proprotein convertase subtilisin/kexin type 9 (PCSK9), 2 transcripts	NM_174936	172	CAGGGGAGGACATCATTGGTG/TTGGCAGAGAAGTGGATCAGTC
SREBP2	Homo sapiens sterol regulatory element binding transcription factor 2 (SREBF2), 2 transcripts	NM_004599	206	AGGAGAAAGGCGGACAACCCATAATA/CCAGCTTCAGCACCATGTTCTC
HNF4α	Homo sapiens hepatocyte nuclear factor 4, alpha (HNF4A), 10 transcripts	NM_000457	145	GGTGCAGGTGAGCTTGGAGGA/GCCGAAGAGCTTGATGAACTGGAT
ACAT2	Homo sapiens acetyl-Coenzyme A acetyltransferase 2 (ACAT2), 2 transcripts	NM_005891	267	CTGTGGCTCCGGAAGATGTGT/CTCCTGTTCTCAAGTAAGCCAAGTG
NPC1L1	Homo sapiens NPC1-like 1 (NPC1L1), 2 transcripts	NM_013389	273	GCTGCTGTTTCTCGCCCTGTT/GGGAACTCTGTGGCATACTGGATCT
ACS1	Homo sapiens acyl-CoA synthetase long-chain family member 1 (ACSL1), 5 transcripts	NM_001995	262	GGCAACCCCAAAGGAGCAATG/TTGGAACCACGGGGAAGACAGT
APOA1	Homo sapiens apolipoprotein A-I (APOA1)	NM_000039	213	TGAAGGACCTGGCCACTGTGTA/GGCCCTCTGTCTCCTTTTCCA
SREBP1c	Homo sapiens sterol regulatory element binding transcription factor 1 (SREBF1), 2 transcripts	NM_004176	283	TGCGGAGAAGCTGCCTATCAACC/TTTGTGGACAGCAGTGCGCAGAC
APOB48R	Homo sapiens apolipoprotein B receptor (APOBR)	NM_018690	135	GCCCAGACCCCAACTAAGCAAC/AGGCTTTACAGACCCCGCGTG

G6PD	Homo sapiens glucose-6-phosphate dehydrogenase (G6PD), nuclear gene encoding mitochondrial protein	NM_000402	121	GATGTCCCCTGTCCCACCAACTCTG/GCAGGGCATTGAGGTTGGGAG
TBP	Homo sapiens TATA box binding protein (TBP)	NM_003194	189	CGGGCACCACTCCACTGTATC/GCTTGGGATTATATTCGGCGTTTC
GUSB	Homo sapiens glucuronidase, beta (GUSB)	NM_000181	130	CGACGAGAGTGCTGGGGAATA/TTGGCTACTGAGTGGGGATACCT
ADNg	Homo sapiens 3-beta-hydroxysteroid dehydrogenase/delta-5-delta-4-isomerase (3-beta-HSD) gene (intron)	M38180	260	GAAGGGCAGAGGTGGAACTAGAA/AACAAAGACCAAAGACCAGTGAGA