Published in final edited form as:

Atherosclerosis. 2017 May ; 260: 27–33. doi:10.1016/j.atherosclerosis.2017.03.015.

Effect of ezetimibe on low- and high-density lipoprotein subclasses in sitosterolemia

Rgia A. Othman^{a,b}, Semone B. Myrie^{a,b,*}, David Mymin^c, Jean-Baptiste Rouillet^{d,e}, Robert D. Steiner^f, and Peter J.H. Jones^{a,b}

^aHuman Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

^bRichardson Center for Functional Foods and Nutraceuticals, University of Manitoba, Winnipeg, Manitoba, Canada

^cInternal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

^dCollege of Pharmacy, Washington State University, Spokane, Washington

^eKnight Cardiovascular Institute, Oregon Health & Science University, Portland, Oregon

^fUniversity of Wisconsin School of Medicine and Public Health, Madison, Wisconsin

Abstract

Background and aims—Sitosterolemia displays high plasma total sterols [high plant sterols (PS) + normal to high total cholesterol (TC)] with normal to moderately elevated low-density lipoprotein (LDL) levels. High LDL, intermediate-density lipoprotein (IDL) and very low-density lipoprotein (VLDL) particles, low high-density lipoprotein (HDL), and increased non-HDL and the ratios of TC and triglycerides (TG) to HDL can increase the risk for atherosclerosis. Ezetimibe (EZE) can reduce plasma PS and TC levels in sitosterolemia, but its effect on lipoprotein subclasses has not been previously reported.

Methods—Sitosterolemia patients (n=8) were taken off EZE for 14 weeks (OFF EZE) and placed on EZE (10 mg/d) for 14 weeks (ON EZE). Serum lipids were measured enzymatically and lipoprotein subclasses were assessed by polyacrylamide gel electrophoresis.

Results—EZE reduced ($p < 0.05$) total sterols ($-12.5 \pm 4.1\%$) and LDL-sterol ($-22.7 \pm 5.7\%$) and its sterol mass of large VLDL ($-24.4 \pm 4.5\%$), VLDL remnants ($-21.1 \pm 7.9\%$) and large IDL ($-22.4 \pm 7.2\%$) compared to OFF EZE. EZE did not affect large LDL subclasses or mean LDL

*Corresponding author: Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba, 196 Innovation Drive, Winnipeg, MB R3T 6C5, Canada. myrie@cc.umanitoba.ca (S. B. Myrie).

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

RAO, PJHJ conceptualized the research question in this report. by SBM, JB, DM, RDS, PJHJ designed the study. RAO, SBM, DM, PJHJ conducted the study. RAO conducted sample analyses and interpretation of the data, and wrote the first draft of the paper. SBM, DM, JB, RDS and PJHJ contributed to interpretation of data, and revision of the manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

particle size (273.8 ± 0.6 vs. 274.6 ± 0.3 Å). EZE increased HDL-sterol ($25.5 \pm 8.0\%$, $p=0.008$) including intermediate ($34 \pm 14\%$, $p=0.02$) and large ($33 \pm 16\%$, $p=0.06$) HDL. EZE reduced non-HDL-sterol ($-21.8 \pm 5.0\%$), total sterols/HDL ($-28.2 \pm 5.5\%$) and TG/HDL ($-27.4 \pm 6.5\%$, all $p<0.01$).

Conclusions—EZE improves VLDL and HDL subfraction distribution, thereby reducing the atherogenic lipid profile, thus providing potential clinical benefit in sitosterolemia beyond TC and PS reduction.

Keywords

LDL cholesterol; LDL subclasses; mean LDL particle size; HDL subclasses; non-HDL cholesterol; ezetimibe; sitosterolemia; sterols

1. Introduction

Sitosterolemia (STSL, OMIM#210250) is a rare autosomal recessive sterol and lipid disorder caused by mutations in either of the two adjacent genes that encode adenosine triphosphate-binding cassette (ABC) half-transporters, ABCG5 or ABCG8, thereby enhancing dietary and biliary retention of cholesterol and plant sterols (PS), and reducing their hepatic excretion into bile for elimination [1]. Major clinical features include premature atherosclerosis and xanthomas [2, 3]. Other complications include hemolytic anemia and macrothrombocytopenia [4, 5]. STSL patients have high plasma total sterols [PS + total cholesterol (TC)], wherein plasma levels of PS are highly elevated and TC ranges from normal to high [6]. Plasma low-density lipoprotein (LDL) sterol levels in STSL remain normal or moderately elevated [1, 7].

Elevated levels of apolipoprotein B-containing lipoproteins (apoB) (i.e. LDL, intermediate-density lipoprotein (IDL) and very low-density lipoprotein (VLDL) particles [8]), non-high-density lipoprotein (HDL) cholesterol and the ratios of TC and triglycerides (TG) to HDL cholesterol are associated with increased atherogenic potential and coronary artery disease (CAD) risk [9–11]. In contrast, increased levels of HDL cholesterol are associated with reduced risk [12]. Lipoproteins are a varied mixture of particles that differ in size, density and lipid content. LDL particles are classified into two phenotypes based on size. Phenotype A is associated with non-atherogenic LDL with particles larger than 268Å and buoyant, while phenotype B is linked to atherogenic LDL with particles smaller than 265Å , called small and dense LDL (sdLDL) [13, 14]. Similarly, the HDL family consists of large, intermediate and small HDL subclasses that are not equally atheroprotective [15]. Available evidence suggests that cardioprotective properties of HDL may be related to particle size and density [16].

Ezetimibe is a sterol absorption inhibitor used for the treatment of STSL by reduction of elevated levels of blood PS, and of hypercholesterolemia by reduction of elevated levels of LDL cholesterol [6, 17]. Thus far, the impact of EZE on serum atherogenic and antiatherogenic lipoprotein (LDL and HDL) subfractions in STSL has not been determined. It is important to investigate the effect of EZE on these lipoprotein subfractions because premature atherosclerosis has been frequently reported in STSL [2, 18]. We thus evaluated

the concentration of serum lipid classes, subclasses (LDL and HDL) and mean LDL particle size as well as lipid markers of cardiovascular risk (non-HDL cholesterol, TC/HDL and TG/HDL) in STSL patients both with and without EZE.

2. Materials and methods

2.1. Study design and population

The design of the study from which these data were taken was previously published [19]. This was a single-site pilot interventional study assessing the effect of ezetimibe (10 mg/day; EZE) on cholesterol and plant sterol metabolism in patients with sitosterolemia. This report describes the effect of EZE on lipids and lipoproteins.

All participants were from Hutterite communities and were identified as having homozygous *ABCG8* S107X mutations (NM_022437.2:c.320C>G). They were all treated with EZE as part of standard of care, and all gave written informed consent. Subjects were taken off their daily treatment with EZE for 14 weeks (OFF EZE phase) after which they resumed EZE at 10 mg/day for 14 weeks (ON EZE phase). Blood collection was performed at baseline, at the end of each phase and in between (not used in this report for lipoprotein analyses). All participants completed the study and no adverse events were reported.

The study was conducted at the Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba, and registered with www.ClinicalTrials.gov as NCT01584206. The study was reviewed and approved by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development and the Biomedical Ethics Board of the University of Manitoba. It was conducted by investigators and trainees of the Sterol and Isoprenoid Research consortium (<https://rarediseasesnetwork.epi.usf.edu/STAIR/>).

2.2. Serum lipid and lipoprotein analysis

Serum TG, total sterols (PS +TC) and HDL sterol concentrations were determined at baseline and 14 weeks OFF EZE and ON EZE by automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). Enzymatic measurement of serum TC in sitosterolemia measures cholesterol plus PS, thus quantitative lipids and lipoprotein should be expressed as serum total sterol and lipoprotein sterol [20], as used for expressing data measured in this report. However, the proportion of total sterol that was PS was determined by gas-liquid chromatography and was previously reported [19] (see Results section). LDL sterol concentrations were calculated using the Friedewald equation [21]. Non-HDL sterol concentrations were calculated by subtracting the HDL sterol value from the total sterol value; here this included VLDL + IDL+ LDL.

2.3. Lipoprotein subfractions analyses

LDL and HDL subfractions analyses were performed on serum samples taken at 14 weeks OFF EZE and ON EZE. The subfractions were detected by polyacrylamide gel electrophoresis using the Lipoprint system (Quantimetrix Corporation, Redondo Beach, CA, USA) according to the manufacturer's instructions. Briefly, 25 µL of serum sample was mixed with 200 µL (LDL subfractions) or 300 µL (HDL subfractions) of Lipoprint Loading

Gel and added to precast 3% polyacrylamide gel tubes. The loading gels were allowed to photopolymerize at room temperature for 30 minutes, and electrophoresis was done for 60 minutes (LDL subfractions) or 50 minutes (HDL subfractions) with 3 mA/gel tube. A quality control sample (provided by the manufacturer) was run with each electrophoresis chamber. Gels were scanned using a ScanMaker 8700 digital scanner (Mikrotek Co, USA). After scanning, retention factor (Rf) or electrophoretic mobility and the area under the curve were calculated qualitatively with the Lipoprint system Templates, and quantitatively with Lipoware software (Quantimetrix, Redondo Beach, CA). LDL lipoprotein subfractions were identified in the sample by their Rf, using VLDL as the starting reference point (Rf = 0.0) and HDL as the leading reference point (Rf = 1.0). The Lipoprint LDL system allows the analysis of 12 lipoprotein subfractions: 1 large VLDL band, 3 IDL midbands: mid-C (VLDL remnants), mid-B (large IDL), mid-A (small IDL), and up to 7 LDL bands: LDL-1 and LDL-2 (large buoyant LDL), LDL-3 to LDL-7 (sdLDL). The mobility of the HDL subfractions was determined using comparisons with LDL/VLDL (Rf = 0.0) and albumin (Rf = 1.0). In between, up to 10 subfractions of HDL were detected; 1 to 3 were large, 4 to 7 were medium and 8 to 10 were small HDL particles. The Lipoware software automatically calculates the amount of sterol (mg/dL) in each subfraction based on the total sterols or HDL sterol concentration of the sample, measured enzymatically. Moreover, the Lipoprint LDL system provides a mean LDL particle size in Angstroms (Å).

2.4. Statistical analysis

All data are presented as mean $\pm$ SEM. Paired Student's t-tests were applied for comparisons between study parameters. Correlations between two parameters were assessed using Pearson correlation analysis. Stepwise multivariate linear regression analysis was used for determining the independent relationship between mean LDL particle size and serum lipids. All reported p-values were calculated as two-sided and statistical significance was assumed at $p < 0.05$. Statistical analyses were performed using SPSS 21.0 (SPSS, Inc, Chicago, Illinois, USA).

4. Results

4.1. Baseline characteristics

Eight STSL patients (5 males and 3 females; age range 16–65 years) were recruited and completed the trial (Table 1). On average, the study subjects were overweight based on body mass index ($25\text{--}29.9\text{ kg/m}^2$). Their blood pressure ranged from 120/80 mmHg to 140/90 mmHg. Fasting TG concentrations were $<150\text{ mg/dL}$. Average total sterols ($218.0 \pm 17.7\text{ mg/dL}$) and LDL sterol ($139.2 \pm 15.1\text{ mg/dL}$) concentrations were borderline high ($200\text{--}239\text{ mg/dL}$) and ($130\text{--}159\text{ mg/dL}$), respectively. Serum HDL sterol concentrations were within normal levels ($>40\text{ mg/dL}$) while serum lipid markers of cardiovascular risk were borderline high for non-HDL sterol ($163.4 \pm 17.0\text{ mg/dL}$), and slightly higher for both total sterols/HDL (4.0 ± 0.3) and TG/HDL (2.2 ± 0.5) than normal limits ($<130\text{ mg/dL}$, and <3.5 and <2.0).

4.2. Effect of ezetimibe on serum PS and lipid profile

For the participants in this study, plasma PS and TC levels were also determined by gas-liquid chromatography method and previously reported [19]. In summary, plasma PS concentrations were 11.8 ± 0.8 mg/dL OFF EZE and 7.5 ± 0.6 mg/dL ON EZE, representing about 5% and 4%, respectively, of the TC levels measured by enzymatic method (241.2 ± 14.0 mg/dL OFF EZE vs. 209.3 ± 12.1 mg/dL ON EZE). TC levels measured by gas-liquid chromatography method were 178.3 ± 13.9 mg/dL OFF EZE and $153.8.3 \pm 10.7$ mg/dL ON EZE. Thus, overall the ratio of PS to TC was reduced by 27% regardless of the method of cholesterol measurement used.

On average TG, total sterols, LDL sterol and HDL concentrations at week 14 OFF EZE (Table 2) were not statistically different from baseline (Table 1), all $p > 0.05$.

EZE therapy for 14 weeks did not significantly change serum levels of TG, but decreased total sterols and LDL sterol, and increased HDL sterol compared to 14 weeks OFF EZE (Table 2).

4.3. Effect of ezetimibe on serum LDL subfractions and mean LDL particle size

The LDL subclasses on the Lipoprint system were resolved into VLDL, IDL midbands C, B and A, LDL subfractions, and HDL band (Supplemental Fig. 1A).

At week 14 OFF EZE, patients had high concentrations of large VLDL (45.5 ± 3.6 mg/dL) and VLDL remnants (IDL midband C, 30.3 ± 3.1 mg/dL), as well as borderline high levels of large IDL (IDL midband B, 18.0 ± 2.4 mg/dL) and small IDL (IDL midband A, 26.9 ± 2.4 mg/dL) (Fig. 1A). Resuming EZE for 14 weeks decreased sterol mass of large VLDL ($-24.4 \pm 4.5\%$, $p=0.002$), VLDL remnants ($-21.1 \pm 7.9\%$, $p=0.036$) and large IDL ($-22.4 \pm 7.2\%$, $p=0.025$) but not small IDL ($-7.7 \pm 5.6\%$, $p=0.207$) compared to 14 weeks OFF EZE.

At week 14 OFF EZE, sterol levels of large, buoyant LDL subclasses: LDL-1 (44.8 ± 3.9 mg/dL) and LDL-2 (13.4 ± 4.3 mg/dL) were within normal levels (< 57 and 30 mg/dL, respectively). EZE did not change LDL-1 ($-8.0 \pm 7.9\%$, $p=0.233$) and LDL-2 ($+34.8 \pm 55.8\%$, $p=0.174$) after 14 weeks (Fig. 1B). Serum sterol concentrations of sdLDL (LDL-3, 1 mg/dL) were detectable only in two patients at 14 weeks OFF EZE and in one patient ON EZE, and remained within normal levels (< 6 mg/dL). Furthermore, other sdLDL subfractions (4 to 7) were not detected in any patient at any time during the study period.

At week 14 OFF EZE, mean LDL particle size was within normal value (> 268 Å, indicating phenotype A). EZE did not change mean LDL particle size after 14 weeks (274.6 ± 0.3 vs. 273.8 ± 0.6 Å, $p=0.175$). Pearson correlation analysis showed that mean LDL particle size negatively correlated with TG ($r=-0.676$, $p=0.066$), total sterols ($r=-0.800$, $p=0.017$) and LDL sterol ($r=-0.739$, $p=0.036$) at week 14 OFF EZE, but such associations were no longer found after EZE treatment (Supplemental Table 1).

4.4. Effect of ezetimibe on serum HDL sterol concentrations and HDL subclasses

The HDL subclasses on the Lipoprint system were resolved into 10 subfractions (Supplemental Fig. 1B). HDL 1–3 subclasses represent the large HDL, commonly referred

to as HDL-2. HDL 4–7 subclasses represent the intermediate HDL, and HDL 8–10 subclasses represent the small HDL, usually signified as HDL-3.

EZE increased serum HDL sterol ($25.5 \pm 8.0\%$, $p=0.008$) compared to 14 weeks OFF EZE (Table 2, Fig. 2A). At week 14 OFF EZE, sterol concentrations of large (18.0 ± 3.0 mg/dL), intermediate (26.0 ± 2.0 mg/dL), and small HDL (6.0 ± 1.0 mg/dL) subclasses were within normal levels (>10 , >22 , <11 mg/dL, respectively) (Fig. 2A). Large and intermediate, but not small HDL, positively correlated with HDL sterol concentrations ($r=0.854$, $p=0.007$ and $r=0.790$, $p=0.020$ vs. $r=0.596$, $p=0.119$), respectively. After 14 weeks, EZE tended to increase ($+32.5 \pm 16.0\%$, $p=0.063$) sterol concentrations of large HDL compared to 14 weeks OFF EZE (Fig. 2A). This increase was mainly due to increases in sterol concentration of HDL-2 ($+43.2 \pm 15.5\%$, $p=0.021$) and HDL-3 ($+36.6 \pm 13.9\%$, $p=0.026$), but not HDL-1 ($+26.2 \pm 19.5\%$, $p=0.342$) (Fig. 2B). Furthermore, the 14-week ON EZE sterol concentration of large HDL subclasses positively correlated ($r=0.915$, $p=0.001$) with those of HDL sterol. Sterol levels of intermediate HDL subclasses (HDL-4 to HDL-7) were also increased with EZE ($+25.1 \pm 12.2\%$, $p=0.032$) (Fig. 2A). This was mainly due to increased HDL-4 ($+32.0 \pm 11.5\%$, $p=0.024$), HDL-5 ($+32.3 \pm 14.0\%$, $p=0.019$), HDL-6 ($+27.0 \pm 16.4\%$, $p=0.054$), but not HDL-7 ($+21.9 \pm 10.8\%$, $p=0.104$) (Fig. 2B). Moreover, the 14-week ON EZE sterol concentration of intermediate HDL subclasses did not correlate ($r=0.425$, $p=0.294$) with those of HDL sterol. After 14 weeks, EZE did not significantly alter sterol concentrations of small HDL subclasses ($+24.3 \pm 13.9\%$, $p=0.111$) (Fig. 2A) despite changes in those of HDL-8 ($+37.5 \pm 15.7\%$, $p=0.033$) but not HDL-9 ($+43.8 \pm 25.8\%$, $p=0.104$) or HDL-10 ($-14.3 \pm 8.6\%$, $p=0.598$) (Fig. 2B). Furthermore, the 14-week ON EZE sterol concentration of small HDL subclasses did not correlate ($r=0.268$, $p=0.521$) with those of HDL sterol. Overall, EZE-induced changes in sterol concentrations of large HDL showed a positive correlation ($r=0.782$, $p=0.022$) with EZE-induced changes in HDL sterol, but not with intermediate ($r=0.596$, $p=0.116$) or small HDL subclasses ($r=0.562$, $p=0.146$). These results suggest that increases in HDL sterol levels with EZE may be due mostly to increased sterol mass of large HDL subclasses.

4.5. Effect of ezetimibe on serum lipid markers of cardiovascular risk

No significant differences were observed between the 14-week OFF EZE serum lipid cardiovascular risk markers (Table 2) including non-HDL sterol, total sterols/HDL and TG/HDL compared to baseline (Table 1). However, EZE therapy for 14 weeks reduced all three parameters compared to 14 weeks OFF EZE (Table 2). At week 14 OFF EZE, Pearson correlation analysis showed that non-HDL sterol, total sterols/HDL and TG/HDL negatively correlated with mean LDL particle size; however, these associations were no longer found after EZE treatment (Supplemental Table 1). The correlations were further tested by stepwise multivariable linear regression analysis and when all serum lipids (total sterols, LDL sterol, HDL sterol, total sterols/HDL and TG/HDL) were included in the multivariate model, only non-HDL sterol (standardized coefficients Beta value = -0.868 ; $r^2=0.754$, $p=0.005$) was the significant predictor of mean LDL particle size (Supplemental Table 2).

5. Discussion

Our results indicated that in STSL subjects, EZE therapy not only reduced serum plant sterols, total sterols and LDL sterol, but also reduced sterol concentrations of large VLDL, VLDL remnants, and IDL without affecting large, buoyant LDL subclasses or mean LDL particle size. Furthermore, EZE is effective at increasing HDL sterol, elevating large and intermediate but not small HDL subclasses. These data indicate that in addition to reducing plant and total sterols, EZE may favorably improve VLDL and HDL subfractions, thus providing potential further cardiovascular protection in STSL patients.

The LDL cholesterol-lowering effect of EZE is well documented in hypercholesterolemic subjects [22]. Likewise, the present study found that EZE reduced serum concentrations of total sterols by 13% and LDL sterol by 20% after 14 weeks. The effect of EZE on lipid and lipid subclasses has been reported in other disease states such as dyslipidemia [23] and type 2 diabetes [24, 25]. Prior to this study, the effects of EZE on LDL and HDL subclasses in STSL had not been elucidated. EZE reduced serum concentrations of TC (−16%), LDL cholesterol (−20%) and VLDL cholesterol (−17%) in primary dyslipidemia after 16 weeks [23]. In the current study, EZE decreased sterol concentrations of large VLDL, VLDL remnants and large IDL by at least 20%, and the decreases in LDL sterol levels may be mainly due to reductions in the sterol carried on the highly atherogenic TG-enriched large VLDL, VLDL remnants and IDL. This finding is clinically relevant because elevated levels of large VLDL increase the risk of experiencing extensive CAD event by four times [26]. VLDL carries most of the circulating TG, and reduction in VLDL might be reflected by decreases in TG levels. In the current study, although EZE was able to reduce sterol concentrations in VLDLs and IDL, these changes were not significantly associated with changes in TG levels. This may be explained by the fact that ON EZE (week 14) we observed large variability in TG levels among the study participants, resulting in changes ranging from reduction of −6% to −20% for 6 participants, and increases of 1% and 35% for 2 participants. It should be noted that we also had the chance to measure serum TG levels at week 12 (not included in this report), and we also observed that 5 patients showed reduction in TG levels while three patients showed increases; thus, again, on average TG levels did not significantly decrease after EZE at week 12. Overall, on average, the changes in TG levels were not significantly different compared with OFF EZE. Sterol content of VLDL, VLDL remnants and large IDL were directly measured by Lipoprint only at week 14, where all patients showed decreases. It has been shown that sterol contents of VLDL remnants and IDL are closely associated with CAD risk, even in patients with normal TG levels [27]. It is believed that EZE decreases cholesterol content in chylomicrons, probably reducing the cholesterol content of large VLDL and thus VLDL remnants [24]. These effects of EZE may also involve a reduction in VLDL production and up-regulation of LDL receptor-mediated clearance of apoB-containing particles [28]. However, we did not measure apoB in this study.

EZE has been found to decrease LDL cholesterol in hypercholesterolemia, mainly due to decreases in cholesterol concentration of large LDL and sdLDL subfractions [29] [23]. Kalogirou et al. observed that EZE reduced serum levels of sdLDL particles (−19%) in hypercholesterolemic subjects, with greater reductions in sdLDL particles (−49%) observed

in subjects with high serum TG values (>151 mg/dL) [23]. Patients with elevated TG levels usually have a predominance of sdLDL particles whereas large, buoyant LDL subfractions preponderate in subjects with normal TG values [23]. The current STSL patients showed the presence of large, buoyant LDL particles that are within normal limits, and almost no sdLDL, along with normal TG values (136 mg/dL). These parameters were not altered with 14 weeks of EZE treatment. In fact, sterol concentrations of sdLDL (LDL-3 LDL-7) were barely detected in the current patients, suggesting a non-atherogenic lipoprotein profile phenotype A (predominance large, buoyant LDL particles) rather than an atherogenic phenotype B (predominance of sdLDL particles). Furthermore, mean LDL particle size is closely and inversely associated with plasma TG levels [30]. The present study results agree with others [29] [31] in showing no effect of EZE on mean LDL particle size in STSL patients who were normotriglyceridemic, and had almost no sdLDL particles.

HDL cholesterol concentrations are inversely associated with cardiovascular risk [32] [12]. Large and intermediate HDL subclasses are associated with decreased coronary heart disease (CHD) risk and are considered atheroprotective [16, 26, 29, 33], while those of small HDL are related to increased CHD risk and are deemed to be atherogenic [26, 33]. In patients with primary dyslipidemia, EZE has been shown to decrease plasma HDL cholesterol, which was mainly due to a reduction in small HDL subclasses, without altering large HDL subclasses [23]. In another study with obese subjects, EZE decreased serum concentrations of intermediate and small HDL particles without affecting large HDL subclasses [34]. In the current study, when patients were 14 weeks OFF EZE, sterol concentration of large and intermediate, but not small, HDL subclasses positively correlated with that of HDL sterol; which may be due to the larger blood proportions (37% and 49%). Changes in sterol concentration of large HDL subclasses with EZE positively correlated with changes in HDL sterol levels, suggesting that increased HDL sterol levels with EZE may be mainly due to a rise in the concentration of large HDL. This finding is clinically important because recent reports showed that only large HDL subclasses have shown inverse correlation with very early CAD [35]. Furthermore, high levels of large HDL subclasses have been associated with lower prevalence of major adverse cardiovascular events in non-treated patients with stable CAD [36]. These reports confirm earlier data by Salonen et al. where serum concentration of HDL cholesterol and large HDL subfractions inversely correlated with the risk of acute myocardial infarction [37], suggesting a potential protective role in ischemic heart disease. Overall, in the current study, increased concentrations of HDL sterol and large and intermediate HDL subclasses may be suggestive of an improvement in HDL function. Improvement in HDL anti-atherogenic function can be achieved by stimulating reverse cholesterol transport (RCT) by increasing the concentrations of apoproteins such as apolipoprotein A1 (apoA1), which is highly capable of accepting cholesterol from cells [38]. In the RCT process, ATP-binding cassette transporter A1 (ABCA1) mediates efflux of cellular cholesterol to lipid-poor apoA-I, the major protein of HDL particles, and ABCG1 enhances efflux to larger HDL particles [39]. However, in this study, we did not measure apoA1 or other apoproteins involved in the RCT process. Thus, no conclusive relationships between HDL changes and improvement in HDL function can be drawn from this study.

Serum non-HDL cholesterol, the sum of cholesterol carried in all potentially atherogenic, apoB-containing lipoproteins, and TC/HDL and TG/HDL are considered markers of the apoB atherogenic lipoprotein profile and cardiovascular risk [9–11]. EZE treatment for 16 weeks reduced serum concentrations of non-HDL cholesterol by 18%, TC/HDL by 28% and TG/HDL by 27%, in patients with primary dyslipidemia [23]. Moreover, non-HDL cholesterol has shown a more close and negative correlation with mean LDL particle size in patients with stable CAD compared to LDL cholesterol [10]. In the present study, although all lipid parameters (total sterols, LDL sterol, total sterols/HDL and TG/HDL) negatively correlated with mean LDL particle size, non-HDL sterol was the only significant independent predictor of the mean LDL particle size. These results suggest that non-HDL cholesterol may be used as a further screening measure that could potentially predict cardiovascular risk better than LDL cholesterol alone.

To our knowledge, this is the first report on the effect of EZE on LDL and HDL subfractions in STSL. The current study was limited by the small sample size (n=8); a large-scale study is needed in the future to confirm the conclusion. Given that we included patients of only a single ethnic group, the Hutterites, all with a single genotype, it would be important to replicate this study in sitosterolemia patients with other genotypes prior to generalizing to other populations. We documented changes in LDL and HDL subfractions after 14 weeks with EZE compared to 14 weeks OFF EZE; however, we did not measure lipoproteins subfractions at baseline; therefore, a comparison with baseline was not feasible. In addition, we used the Lipoprint system to perform lipoprotein analysis and the results should be verified by other methodologies. Previous studies have assessed lipoprotein fractions in STSL subjects using ultracentrifugation and agarose gel electrophoresis [3, 20]. Thus, future research should compare the methods.

In conclusion, the current study showed that in STSL subjects, beyond reducing plant and total sterols and LDL sterol concentrations, EZE is also able to improve lipoprotein distribution. EZE reduces sterol carried on proatherogenic large VLDL, VLDL remnants and IDL without affecting large, buoyant LDL subclasses or mean LDL particle size. EZE increases HDL levels due to an increase in intermediate and large HDL subclasses without affecting small HDL and improves the atherogenic lipid profile by reducing lipid markers of cardiovascular risk (non-HDL sterol, total sterols/HDL and TG/HDL). Given that STSL patients have a strong propensity toward premature coronary atherosclerosis, these findings have significant clinical implication, showing that STSL patients gain additional benefit from EZE treatment beyond plant sterol reduction, and may potentially experience cardioprotective benefits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support

Supported by the Canadian Institutes of Health Research (MOP12339). The Sterol and Isoprenoid Research (STAIR; U54HD061939) Consortium is a part of the National Institutes of Health (NIH) Rare Diseases Clinical Research Network, supported through collaboration between the NIH Office of Rare Diseases Research at the National Center for Advancing Translational Science, and National Institute of Child Health and Human Development. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

We are grateful to the patients for their support.

References

1. Salen G, et al. Sitosterolemia. *J Lipid Res.* 1992; 33(7):945–55. [PubMed: 1431587]
2. Mymin D, et al. Image in cardiovascular medicine. Aortic xanthomatosis with coronary ostial occlusion in a child homozygous for a nonsense mutation in ABCG8. *Circulation.* 2003; 107(5): 791. [PubMed: 12578886]
3. Kwiterovich PO Jr, et al. Hyperapobetalipoproteinaemia in two families with xanthomas and phytosterolaemia. *Lancet.* 1981; 1(8218):466–9. [PubMed: 6110091]
4. Wang Z, et al. Specific macrothrombocytopenia/hemolytic anemia associated with sitosterolemia. *Am J Hematol.* 2014; 89(3):320–4. [PubMed: 24166850]
5. Othman RA, Myrie SB, Jones PJ. Non-cholesterol sterols and cholesterol metabolism in sitosterolemia. *Atherosclerosis.* 2013; 231(2):291–9. [PubMed: 24267242]
6. Farnier M, et al. Efficacy and safety of the coadministration of ezetimibe with fenofibrate in patients with mixed hyperlipidaemia. *Eur Heart J.* 2005; 26(9):897–905. [PubMed: 15781429]
7. Park JH, et al. Sitosterolemia presenting with severe hypercholesterolemia and intertriginous xanthomas in a breastfed infant: case report and brief review. *J Clin Endocrinol Metab.* 2014; 99(5): 1512–8. [PubMed: 24423340]
8. Carmena R, Duriez P, Fruchart JC. Atherogenic lipoprotein particles in atherosclerosis. *Circulation.* 2004; 109(23 Suppl 1):III2–7. [PubMed: 15198959]
9. da Luz PL, et al. High ratio of triglycerides to HDL-cholesterol predicts extensive coronary disease. *Clinics (Sao Paulo).* 2008; 63(4):427–32. [PubMed: 18719750]
10. Zhu CG, et al. Circulating non-HDL-C levels were more relevant to atherogenic lipoprotein subfractions compared with LDL-C in patients with stable coronary artery disease. *J Clin Lipidol.* 2015; 9(6):794–800. [PubMed: 26687700]
11. Lemieux I, et al. Total cholesterol/HDL cholesterol ratio vs LDL cholesterol/HDL cholesterol ratio as indices of ischemic heart disease risk in men: the Quebec Cardiovascular Study. *Arch Intern Med.* 2001; 161(22):2685–92. [PubMed: 11732933]
12. Chapman MJ, et al. Raising high-density lipoprotein cholesterol with reduction of cardiovascular risk: the role of nicotinic acid--a position paper developed by the European Consensus Panel on HDL-C. *Curr Med Res Opin.* 2004; 20(8):1253–68. [PubMed: 15324528]
13. Rosenson RS, Otvos JD, Freedman DS. Relations of lipoprotein subclass levels and low-density lipoprotein size to progression of coronary artery disease in the Pravastatin Limitation of Atherosclerosis in the Coronary Arteries (PLAC-I) trial. *Am J Cardiol.* 2002; 90(2):89–94. [PubMed: 12106834]
14. Gardner CD, Fortmann SP, Krauss RM. Association of small low-density lipoprotein particles with the incidence of coronary artery disease in men and women. *JAMA.* 1996; 276(11):875–81. [PubMed: 8782636]
15. Kontush A, Chapman MJ. Antiatherogenic small, dense HDL--guardian angel of the arterial wall? *Nat Clin Pract Cardiovasc Med.* 2006; 3(3):144–53. [PubMed: 16505860]
16. Arsenault BJ, et al. HDL particle size and the risk of coronary heart disease in apparently healthy men and women: the EPIC-Norfolk prospective population study. *Atherosclerosis.* 2009; 206(1): 276–81. [PubMed: 19268944]
17. Mikhailidis DP, et al. The use of ezetimibe in achieving low density lipoprotein lowering goals in clinical practice: position statement of a United Kingdom consensus panel. *Curr Med Res Opin.* 2005; 21(6):959–69. [PubMed: 15969896]

18. Salen G, et al. Lethal atherosclerosis associated with abnormal plasma and tissue sterol composition in sitosterolemia with xanthomatosis. *J Lipid Res.* 1985; 26(9):1126–33. [PubMed: 4067433]
19. Othman RA, et al. Ezetimibe reduces plant sterol accumulation and favorably increases platelet count in sitosterolemia. *J Pediatr.* 2015; 166(1):125–31. [PubMed: 25444527]
20. Beaty TH, et al. Genetic Analysis of Plasma Sitosterol, Apoprotein B and Lipoproteins in a Large Amish Pedigree with Sitosterolemia. *Am J Hum Genet.* 1986; 38:492–504. [PubMed: 3706300]
21. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem.* 1972; 18(6):499–502. [PubMed: 4337382]
22. Pandor A, et al. Ezetimibe monotherapy for cholesterol lowering in 2,722 people: systematic review and meta-analysis of randomized controlled trials. *J Intern Med.* 2009; 265(5):568–80. [PubMed: 19141093]
23. Kalogirou M, et al. Effect of ezetimibe monotherapy on the concentration of lipoprotein subfractions in patients with primary dyslipidaemia. *Curr Med Res Opin.* 2007; 23(5):1169–76. [PubMed: 17519084]
24. Bozzetto L, et al. Ezetimibe beneficially influences fasting and postprandial triglyceride-rich lipoproteins in type 2 diabetes. *Atherosclerosis.* 2011; 217(1):142–8. [PubMed: 21481394]
25. Sakamoto K, et al. Effect of Ezetimibe on LDL-C Lowering and Atherogenic Lipoprotein Profiles in Type 2 Diabetic Patients Poorly Controlled by Statins. *PLoS One.* 2015; 10(9):e0138332. [PubMed: 26398887]
26. Freedman DS, et al. Relation of lipoprotein subclasses as measured by proton nuclear magnetic resonance spectroscopy to coronary artery disease. *Arterioscler Thromb Vasc Biol.* 1998; 18(7):1046–53. [PubMed: 9672064]
27. Masuoka H, et al. Predictive value of remnant-like particle cholesterol as an indicator of coronary artery stenosis in patients with normal serum triglyceride levels. *Intern Med.* 2000; 39(7):540–6. [PubMed: 10888209]
28. Telford DE, et al. The molecular mechanisms underlying the reduction of LDL apoB-100 by ezetimibe plus simvastatin. *J Lipid Res.* 2007; 48(3):699–708. [PubMed: 17130282]
29. Tribble DL, et al. Effects of fenofibrate and ezetimibe, both as monotherapy and in coadministration, on cholesterol mass within lipoprotein subfractions and low-density lipoprotein peak particle size in patients with mixed hyperlipidemia. *Metabolism.* 2008; 57 (6):796–801. [PubMed: 18502262]
30. McNamara JR, et al. Change in LDL particle size is associated with change in plasma triglyceride concentration. *Arterioscler Thromb.* 1992; 12(11):1284–90. [PubMed: 1420088]
31. Ose L, et al. Effects of ezetimibe/simvastatin on lipoprotein subfractions in patients with primary hypercholesterolemia: an exploratory analysis of archived samples using two commercially available techniques. *Clin Ther.* 2007; 29(11):2419–32. [PubMed: 18158082]
32. Tuteja S, Rader DJ. High-density lipoproteins in the prevention of cardiovascular disease: changing the paradigm. *Clin Pharmacol Ther.* 2014; 96(1):48–56. [PubMed: 24713591]
33. Asztalos BF, et al. High-density lipoprotein subpopulation profile and coronary heart disease prevalence in male participants of the Framingham Offspring Study. *Arterioscler Thromb Vasc Biol.* 2004; 24(11):2181–7. [PubMed: 15388521]
34. Nakou ES, et al. The effect of orlistat and ezetimibe, alone or in combination, on serum LDL and small dense LDL cholesterol levels in overweight and obese patients with hypercholesterolaemia. *Curr Med Res Opin.* 2008; 24(7):1919–29. [PubMed: 18513463]
35. Zhang Y, et al. HDL subfractions and very early CAD: novel findings from untreated patients in a Chinese cohort. *Sci Rep.* 2016; 6:30741. [PubMed: 27489174]
36. Li JJ, et al. Large HDL Subfraction But Not HDL-C Is Closely Linked With Risk Factors, Coronary Severity and Outcomes in a Cohort of Nontreated Patients With Stable Coronary Artery Disease: A Prospective Observational Study. *Medicine (Baltimore).* 2016; 95(4):e2600. [PubMed: 26825910]

37. Salonen JT, et al. HDL, HDL2, and HDL3 subfractions, and the risk of acute myocardial infarction. A prospective population study in eastern Finnish men. *Circulation*. 1991; 84(1):129–39. [PubMed: 2060089]
38. Parhofer KG. Increasing HDL-cholesterol and prevention of atherosclerosis: A critical perspective. *Atheroscler Suppl*. 2015; 18:109–11. [PubMed: 25936313]
39. Watanabe H, et al. Decreased high-density lipoprotein (HDL) particle size, prebeta-, and large HDL subspecies concentration in Finnish low-HDL families: relationship with intima-media thickness. *Arterioscler Thromb Vasc Biol*. 2006; 26(4):897–902. [PubMed: 16469947]

Key Highlights

- Ezetimibe (EZE) improves serum sterols and lipoprotein levels in sitosterolemia.
- VLDL, IDL, and medium and large HDL subclasses increased with EZE.
- EZE had no impact on LDL subclasses and particle size in sitosterolemia.

Fig. 1. Concentrations of LDL sterol and LDL subfractions at 14 weeks off and 14 weeks on ezetimibe in sitosterolemia patients. (A) VLDL and Midband IDL concentrations, and (B) LDL levels and LDL subfractions; LDL3 was detectable only in two patients from samples of 14 weeks OFF EZE and in one patient from samples of 14 weeks ON EZE. Values are means $\pm$ SEM ($n = 8$). ND, not detectable. * $p < 0.05$, ** $p < 0.01$.

Fig. 2.

Concentrations of HDL sterol and HDL subfractions at 14 weeks off and 14 weeks on ezetimibe in sitosterolemia patients.

(A) HDL sterol and the three HDL subclasses, and (B) the lipoprotein subfractions in the three HDL subclasses. Values are means $\pm$ SEM ($n = 8$). * $p < 0.05$, ** $p < 0.01$.

Table 1

Baseline characteristics of sitosterolemia patients.

Variables	Mean $\pm$ SEM	Range
Age (years)	28.0 $\pm$ 5.5	(16.0 – 56.0)
Gender (n: male, female)	(5, 3)	
Body mass index (kg/m ²)	25.7 $\pm$ 2.0	(17.8 – 34.3)
Systolic blood pressure (mmHg)	134.1 $\pm$ 8.0	(107.0 – 169.0)
Diastolic blood pressure (mmHg)	81.5 $\pm$ 3.4	(66.0 – 93.0)
TG (mg/dL)	117.6 $\pm$ 25.5	(57.6 – 287.0)
Total sterols (mg/dL)	218.0 $\pm$ 17.7	(150.8 – 286.2)
LDL sterol (mg/dL)	139.2 $\pm$ 15.1	(83.8 – 194.5)
HDL sterol (mg/dL)	54.6 $\pm$ 2.4	(50.3 – 69.6)
Non-HDL sterol (mg/dL)	163.4 $\pm$ 17.0	(96.7 – 216.6)
Total sterols/HDL	4.0 $\pm$ 0.3	(2.8 – 4.9)
TG/HDL	2.2 $\pm$ 0.5	(1.0 – 5.7)

HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides.

Table 2

Serum lipid profile and some cardiovascular risk markers at 14 weeks off and 14 weeks on ezetimibe in sitosterolemia patients.

Parameters	Normal levels	14 weeks OFF EZE	14 weeks ON EZE	Mean % change	p-value
Lipid profile					
TG (mg/dL)	< 150	136.1 ± 19.7	128.5 ± 28.3	-10.7 ± 7.7	0.540
Total sterols (mg/dL)	< 200	241.2 ± 14.0	209.3 ± 12.1	-12.5 ± 4.1	0.023
LDL sterol (mg/dL)	< 150	164.0 ± 13.4	125.7 ± 12.1	-22.7 ± 5.7	0.008
VLDL (mg/dL)	22	45.5 ± 3.6	34.4 ± 3.5	-24.4 ± 4.5	0.002
HDL sterol (mg/dL)	> 40-59	49.9 ± 4.0	60.9 ± 3.0	+25.5 ± 8.0	0.008
Other CVD risk markers					
Non-HDL sterol (mg/dL)	< 130	191.4 ± 14.5	148.4 ± 12.5	-21.8 ± 5.0	0.006
Total sterols/HDL	< 3.5	5.1 ± 0.5	3.5 ± 0.3	-28.2 ± 5.5	0.004
TG/HDL	< 2.0	2.9 ± 0.5	2.2 ± 0.6	-27.4 ± 6.5	0.009

Values are means ± SEM (n = 8). Changes between 14 weeks OFF EZE and 14 weeks ON EZE were tested for significance by paired t-test. CVD, cardiovascular disease; EZE, ezetimibe; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ND, not detectable; TG, triglycerides; VLDL, very-low-density lipoprotein.