Ezetimibe Increases Endogenous Cholesterol Excretion in Humans

Xiaobo Lin, Susan B. Racette, Lina Ma, Michael Wallendorf, Richard E. Ostlund Jr

Objective—Ezetimibe improves cardiovascular outcomes when added to optimum statin treatment. It lowers low-density lipoprotein cholesterol and percent intestinal cholesterol absorption, but the exact cardioprotective mechanism is unknown. We tested the hypothesis that the dominant effect of ezetimibe is to increase the reverse transport of cholesterol from rapidly mixing endogenous cholesterol pool into the stool.

Approach and Results—In a randomized, placebo-controlled, double-blind parallel trial in 24 healthy subjects with low-density lipoprotein cholesterol 100 to 200 mg/dL, we measured cholesterol metabolism before and after a 6-week treatment period with ezetimibe 10 mg/d or placebo. Plasma cholesterol was labeled by intravenous infusion of cholesterol-4, in a lipid emulsion and dietary cholesterol with cholesterol-4, and sitostanol-4, solubilized in oil. Plasma and stool samples collected during a cholesterol- and phytosterol-controlled metabolic kitchen diet were analyzed by mass spectrometry. Ezetimibe reduced intestinal cholesterol absorption efficiency 30±4.3% (SE, P<0.0001) and low-density lipoprotein cholesterol 19.8±1.9% (P=0.0001). Body cholesterol pool size was unchanged, but fecal endogenous cholesterol excretion increased 66.6±12.2% (P<0.0001) and percent cholesterol excretion from body pools into the stool increased 74.7±14.3% (P<0.0001), whereas plasma cholesterol turnover rose 26.2±3.6% (P=0.0096). Fecal bile acids were unchanged.

Conclusions—Ezetimibe increased the efficiency of reverse cholesterol transport from rapidly mixing plasma and tissue pools into the stool. Further work is needed to examine the potential relation of reverse cholesterol transport and whole body cholesterol metabolism to coronary events and the treatment of atherosclerosis.


Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:990-996. DOI: 10.1161/ATVBHA.117.309119.)

Key Words: cholesterol ■ clinical trial ■ ezetimibe ■ intestinal elimination ■ mass spectrometry ■ physiology

Statins inhibit cholesterol biosynthesis and are highly effective in reducing plasma low-density lipoprotein (LDL) cholesterol levels and cardiovascular disease risk.1 Despite these effects, cardiovascular disease remains the leading cause of death in the United States and other Western countries.2 Thus, complementary strategies are needed to further reduce risk.

Inhibiting intestinal cholesterol absorption represents another approach for LDL cholesterol lowering, which also affects whole body cholesterol metabolism. The drug ezetimibe reduces intestinal cholesterol absorption by targeting NPC1L1 (Niemann-Pick C1-like 1), a sterol transporter expressed in the apical membrane of enterocytes.3,4 As a result, less cholesterol is delivered to the liver, thereby upregulating LDL receptors and reducing LDL cholesterol.5 When given to patients with primary hypercholesterolemia, ezetimibe (10 mg/d) reduces LDL cholesterol 15% to 20%.6,7 Hepatic NPC1L1 is a second target of ezetimibe.8 Even though ezetimibe may increase cholesterol excretion by inhibiting intestinal NPC1L1 alone (in rodents, eg), reducing both hepatic NPC1L1 and intestinal NPC1L1 is expected to maximize endogenous cholesterol excretion in species, such as humans, where NPC1L1 is expressed both in enterocytes and in hepatocytes. Hepatic NPC1L1 reclaims cholesterol from the bile back into the liver, inhibiting biliary cholesterol secretion.9 Ezetimibe, by inhibiting hepatic NPC1L1, stimulates biliary cholesterol secretion; simultaneous inhibition of intestinal NPC1L1 by ezetimibe facilitates excretion of this biliary cholesterol in the stool.

Reverse cholesterol transport (RCT), the process of removing excess cholesterol from peripheral tissues to the rapidly mixing plasma and tissue pool and then directing it to the intestine for excretion,10,11 has been an area of intense research because it is another mechanism with the potential to reduce cardiovascular disease risk.12 The terminal portion of RCT involves 2 pathways: biliary secretion and an intestinal phase where cholesterol is both absorbed from the lumen and directly secreted into the lumen. The latter process
is known as transintestinal cholesterol excretion (TICE).\textsuperscript{13} Cholesterol from both pathways arrives in the small intestine and is excreted into the stool. Inhibiting intestinal cholesterol absorption not only reduces plasma LDL cholesterol levels but also significantly increases total fecal cholesterol excretion.\textsuperscript{14,15} Because the majority of intestinal cholesterol comes from endogenous cholesterol, ezetimibe is expected to promote fecal excretion of endogenous cholesterol (FEEC) by inhibiting intestinal NPC1L1, as suggested in animal studies.\textsuperscript{16}

Two published clinical studies using pioneering mass spectroscopic methodologies have examined changes in body cholesterol metabolism associated with ezetimibe treatment; both support the idea that ezetimibe increases endogenous cholesterol excretion.\textsuperscript{15,17} However, this conclusion is tempered by limitations present in one or both those trials, including lack of a control group, lack of concurrent ezetimibe treatment during metabolic measurements, collection of stool samples without control of either dietary cholesterol or phytosterol intake, lack of measurement of intestinal cholesterol absorption efficiency or total endogenous cholesterol excretion, and a focus on analyses performed during rapid changes in cholesterol enrichment, where repeatability of calculated parameters is limited. In the present work, we tried to optimize the experimental conditions while emphasizing the critical roles of the rapidly mixing endogenous cholesterol pool and the intestine in the action of ezetimibe. Using a well-defined intravenous cholesterol tracer and oral tracers, we carefully identified the origins of fecal cholesterol as endogenous, dietary, or unlabeled cholesterol arising from newly synthesized, nonequilibrated cholesterol.\textsuperscript{18} Measurements were made while the subjects were consuming a metabolic kitchen diet under near steady-state conditions, where results in the placebo group were reproducible. We advanced the notion that percent cholesterol excretion, the percentage of the rapidly mixing cholesterol pool excreted in the stool daily, is helpful in understanding and quantifying the effect of ezetimibe on whole body cholesterol metabolism.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Subjects

As shown in Figure 1, 26 subjects were randomized and 24 completed the protocol (12 ezetimibe, 12 placebo). Subjects included 15 females and 9 males with mean age of 55.6±2.6 years. Table 1 shows the subject characteristics on entry into the study.

#### Cholesterol Metabolism

Table 2 shows the results of cholesterol metabolic measurements for the pre- and post-treatment periods of placebo and ezetimibe groups and the respective absolute differences in pre–post change between placebo and ezetimibe groups. Ezetimibe treatment increased fecal total neutral sterol excretion +0.37±0.08 g/d relative to placebo (Table 2) and 54.0% (Figure 2A). In addition, ezetimibe increased FEEC (+0.31±0.06 g/d; Table 2; 66.6±12.2%; Figure 2B) and dietary cholesterol (+0.038±0.006 g/d; Table 2; 52.0±8.5%; Figure 2C). Endogenous cholesterol was the major contributor to the

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>FEEC</td>
<td>fecal excretion of endogenous cholesterol</td>
</tr>
<tr>
<td>mRCT</td>
<td>macrophage-specific reverse cholesterol transport</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-Pick C1-like 1</td>
</tr>
<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
</tr>
<tr>
<td>TICE</td>
<td>transintestinal cholesterol excretion</td>
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**Figure 1.** CONSORT (Consolidated Standards of Reporting Trials) flow diagram. Flow of subjects throughout the trial. All completed subjects were included in the analyses.
increase in fecal sterol excretion, accounting for 86.6±11.9% of the increase (Figure 2D).

Ezetimibe did not alter excretion of unlabeled cholesterol, which represents newly synthesized cholesterol that has not equilibrated with tracer, or excretion of bile acids (Table 2). Furthermore, the rapidly mixing pool of body cholesterol was not affected by ezetimibe (Table 2). However, ezetimibe reduced relative cholesterol-Δ, enrichment by 0.07±0.004 (Table 2) or 26.2±3.6% (Figure 3A), indicating an increased turnover of body cholesterol by ezetimibe. In addition, ezetimibe treatment increased percent cholesterol excretion by +1.19±0.23 percentage points (Table 2) or 74.7±14.3% (P<0.0001; Figure 3B). The treatment effect on intestinal cholesterol absorption was ~18.9±3.2 percentage points (Table 2) or -30.0±4.3% with ezetimibe treatment relative to the pretreatment period.

**Plasma Lipids and Lipoproteins**

Ezetimibe treatment significantly reduced plasma LDL cholesterol levels by 28.2±6.3 mg/dL (Table 2) or 19.8±1.9% (P=0.0001) relative to the pretreatment and total cholesterol by 33.9±8.4 mg/dL (Table 2) or 15±0.02% (P=0.0003). Plasma triglyceride and HDL cholesterol concentrations were not affected.

**Plasma Noncholesterol Sterols**

Ezetimibe significantly reduced plasma concentrations of total phytosterols and the 2 major individual phytosterols: campesterol and sitosterol (Table 2). Ezetimibe increased plasma lathosterol/total cholesterol ratio, but did not affect plasma 5α-cholestanol to total cholesterol ratio (Table 2).

**Discussion**

The major finding of our study is that ezetimibe increased FEEC and endogenous cholesterol turnover without affecting the size of the rapidly mixing body cholesterol pool. As expected, ezetimibe reduced plasma total cholesterol (15.0%) and LDL cholesterol (19.8%) without affecting the concentrations of plasma HDL cholesterol or triglycerides. Ezetimibe also reduced intestinal cholesterol absorption (30.0%). The effect of ezetimibe on whole body cholesterol metabolism was much larger than its effect on LDL cholesterol and intestinal cholesterol absorption. Ezetimibe increased fecal excretion of total neutral sterols by 54.0%, which is consistent with a 64.0% increase from our previous study of ezetimibe. Both the amount and the efficiency of endogenous cholesterol excretion from the rapidly mixing cholesterol plasma and tissue pool into the stool increased. FEEC rose 66.6% and percent cholesterol excretion rose by 74.7%.

Fecal neutral sterols arise from endogenous cholesterol in the rapidly mixing body pool, unabsorbed dietary cholesterol, and unlabeled newly synthesized cholesterol, all of which might potentially be altered by ezetimibe and contribute to the rise in total fecal sterol output observed. In the present study, the use of oral and intravenous cholesterol tracers revealed that the increase in FEEC accounted for 86.6% of the increase in fecal excretion of total neutral sterols because of ezetimibe. In contrast, excretion of unabsorbed dietary cholesterol and unlabeled cholesterol accounted for only 11.0% and 2.4%, respectively. The exclusion of unlabeled newly synthesized cholesterol as a small component of fecal cholesterol with ezetimibe treatment demonstrates that the effect of ezetimibe is not simply because of a futile cycle characterized by increased local intestinal synthesis of cholesterol. Moreover, the data show that RCT from liver, plasma, and intestine into the stool rises.

Endogenous cholesterol is likely secreted into the intestine both in bile and directly through TICE. The intestinal sterol transporter NPC1L1, located in the apical membrane of enterocytes, mediates both dietary and endogenous cholesterol uptake into the enterocyte. Because the proximal small intestine is actively involved in TICE, the endogenous cholesterol available for reuptake into the enterocyte comprises not only biliary cholesterol but also cholesterol secreted through TICE. By inhibiting NPC1L1, ezetimibe reduces the intestinal reabsorption of dietary, biliary, and TICE-derivated cholesterol, leading to losses in the stool. Ezetimibe may have stimulated both biliary RCT and nonbiliary RCT (ie, TICE) in the present study. The action of ezetimibe to increase fecal excretion of labeled cholesterol from macrophages (macrophage-specific RCT [mRCT]) by inhibiting intestinal NPC1L1 was demonstrated in mice, a species in which NPC1L1 is not measurably expressed in the liver. Whether this happens in humans cannot be determined from our data. More recently, it has been demonstrated in vitro that ezetimibe may promote cholesterol efflux from the brush-border membrane of enterocytes into the lumen, which may help the drug both reduce intestinal cholesterol absorption and enhance cellular cholesterol flux back to the intestinal lumen.

In humans, NPC1L1 also is expressed in the liver, where NPC1L1 inhibits biliary cholesterol secretion. Ezetimibe targets hepatic NPC1L1, stimulating biliary cholesterol secretion. The additional potential biliary cholesterol resulting from the inhibition of hepatic NPC1L1 arrives in the small intestine, where it is expected to be excreted in the stool.
because intestinal NPC1L1 is inhibited simultaneously. In the current study, the increase in FEEC by ezetimibe may reflect increased biliary cholesterol secretion (because of inhibition of hepatic NPC1L1) facilitated by reduced reabsorption of biliary cholesterol (because of inhibition of intestinal NPC1L1). The unique dual-target mechanism of ezetimibe may maximize RCT by the combined inhibition of both hepatic NPC1L1 and intestinal NPC1L1 and thus provide better atheroprotection in species with NPC1L1 expressed in both the liver and the intestine, such as humans. The importance of inhibiting intestinal cholesterol absorption is supported by 2 elegant studies with LDL receptor knockout mice that overexpressed hepatic ABCG5 and ABCG8 and had increased biliary cholesterol secretion.22 However, no effects were observed on intestinal cholesterol absorption, fecal sterol excretion, or aortic atherosclerosis with functional intestinal NPC1L1.22 In the same transgenic mouse model, ezetimibe inhibited intestinal cholesterol absorption, leading to an increase in fecal sterol excretion and a reduction in proximal aortic atherosclerosis.23 These results suggest that the terminal portion of the RCT pathway focused on liver and intestine may be important in determining cardiovascular disease risk.

It is not clear from our current study whether ezetimibe mainly increased biliary secretion or TICE. In mice, ezetimibe increased mRCT by inhibiting intestinal NPC1L1, which required efficient biliary cholesterol secretion.24 Similarly, in hamsters, ezetimibe has been demonstrated to increase mRCT mainly through increased biliary cholesterol secretion and independent of TICE.25 In contrast, ezetimibe

| Table 2. Cholesterol Metabolic Measurements Before and After Treatment |
|--------------------------|--------------------------|--------------------------|
|                          | Placebo                  | Ezetimibe (10 mg/d) | Treatment Difference |
| Cholesterol metabolism, g/d |                          |                      |                       |
| Total fecal cholesterol  | 0.82±0.07                | 0.81±0.08            | 0.71±0.04             | 1.07±0.08†            | 0.37±0.08‡            |
| Endogenous origin        | 0.59±0.08                | 0.58±0.08            | 0.51±0.05             | 0.82±0.06†            | 0.31±0.06‡            |
| Dietary origin           | 0.090±0.010              | 0.090±0.010          | 0.080±0.010           | 0.120±0.010†          | 0.038±0.006‡          |
| Unlabeled                | 0.15±0.06                | 0.14±0.05            | 0.12±0.03             | 0.13±0.06             | NS                    |
| Percent cholesterol excretion, %/d | 1.85±0.25                | 1.86±0.23            | 1.78±0.17             | 2.99±0.22†            | 1.19±0.23‡            |
| Total fecal bile acids, g/d | 0.51±0.02                | 0.63±0.06            | 0.51±0.02             | 0.66±0.06*            | NS                    |
| Cholesterol rapidly mixing pool size, g/d | 32.0±1.9                | 31.1±1.5             | 29.0±0.9              | 27.5±1.0              | NS                    |
| Plasma relative cholesterol d7 enrichment | 0.28±0.02                | 0.27±0.01            | 0.32±0.02             | 0.23±0.01†            | −0.07±0.004‡          |
| Percent cholesterol absorption, % | 61.7±3.2                | 61.6±3.3             | 61.5±2.6              | 42.5±2.7†             | −18.9±3.2‡            |
| Plasma lipids and lipoproteins, mg/dL |
| Total cholesterol        | 212.3±6.3                | 213.0±6.7            | 222.6±13.1            | 189.3±9.9†            | −33.9±8.4‡            |
| LDL cholesterol          | 141.2±5.3                | 139.2±6.0            | 144.6±10.9            | 114.4±8.7†            | −28.2±6.3‡            |
| HDL cholesterol          | 48.6±3.3                 | 46.2±3.5             | 55.2±2.9              | 55.7±3.2              | NS                    |
| Triglycerides            | 121.6±11.5               | 141.1±14.4           | 109.9±8.9             | 106.3±9.6             | NS                    |
| Plasma noncholesterol sterols, µg/mg |
| Cholestanol/total cholesterol | 1.20±0.09                | 1.26±0.08            | 1.49±0.12             | 1.41±0.08             | NS                    |
| Lathosterol/total cholesterol | 1.18±0.12                | 1.06±0.13            | 1.00±0.09             | 1.34±0.11†            | 0.45±0.08‡            |
| Total phytosterols/total cholesterol | 1.92±0.34                | 1.89±0.32            | 2.46±0.26             | 1.42±0.14†            | −1.01±0.18‡           |
| Campesterol/total cholesterol | 0.90±0.16                | 0.87±0.17            | 1.22±0.14             | 0.61±0.08†            | −0.57±0.10‡           |
| Sitosterol/total cholesterol | 0.97±0.18                | 0.98±0.15            | 1.18±0.11             | 0.77±0.06†            | −0.43±0.10‡           |
| Stigmasterol/total cholesterol | 0.045±0.005              | 0.040±0.004          | 0.054±0.006           | 0.038±0.003‡          | NS                    |

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and NS, not significant. All values are means±SE, n=12 subjects per group. Significance of pre- to post-differences in each group are indicated by * for P<0.05 and † for P<0.01.

Cholesterol metabolism: Total fecal cholesterol was measured as the sum of intact cholesterol and its bacterial metabolites, coprostanol and coprostanone. It is composed of material of endogenous origin labeled with cholesterol-d7, dietary origin measured with cholesterol-d5, or unlabeled cholesterol. Percent cholesterol excretion is the percentage of the rapidly mixing cholesterol pool per day excreted in the feces. Plasma relative cholesterol d7 enrichment for pretreatment is calculated as (plasma cholesterol d7 enrichment on day 15−plasma cholesterol d7 enrichment on day 1)/(plasma cholesterol d7 enrichment on day 2−plasma cholesterol d7 enrichment on day 1); for post-treatment, it is calculated as (plasma cholesterol d7 enrichment on day 57−plasma cholesterol d7 enrichment on day 43)/(plasma cholesterol d7 enrichment on day 44−plasma cholesterol d7 enrichment on day 43).

Treatment difference is the absolute difference in pre–post change between placebo and ezetimibe groups, ‡P<0.01.
enhanced endogenous cholesterol excretion in humans predominantly by increasing TICE, mediated by intestinal ABCG5/ABCG8.26

It has been demonstrated that ezetimibe increases cholesterol synthesis in humans.5,15 In the present study, ezetimibe increased total fecal sterols, as well as plasma lathosterol/total cholesterol ratio, a biomarker for cholesterol synthesis.27 This result suggests that increased plasma lathosterol may result from relative cholesterol deficiency and does not necessarily indicate a primary disorder of cholesterol overproduction. Likewise, the observed reduction in phytosterol/cholesterol levels likely was because of reduced absorption rather than a change in dietary phytosterol intake.

Even though mRCT represents only a small fraction of whole body cholesterol flux, there is interest in targeting mRCT in the prevention or regression of atherosclerosis. Several studies have demonstrated such an effect mediated by ezetimibe in mice16,20,24 and in hamsters.25 In the present study, cholesterol fluxes from macrophages or peripheral tissues into plasma were not assessed directly. However, ezetimibe in the present study may have increased cholesterol efflux from extrahepatic tissues, including macrophages. The immediate source of endogenous cholesterol excretion is from the rapidly mixing cholesterol pool. The size of this rapid pool was unchanged in the present study, suggesting that cholesterol efflux from peripheral tissues or cholesterol biosynthesis was responsible for the increase in cholesterol excretion. Ezetimibe significantly increased the turnover of cholesterol in body pools and plasma. In mice, ezetimibe reduced plasma LDL cholesterol mainly by reducing hepatic lipoprotein secretion and, to a lesser extent, by increasing lipoprotein clearance mediated by the upregulated LDL receptors.28 In insulin-resistant obese humans, a weight loss diet alone and a weight loss diet plus ezetimibe both reduced VLDL-apoB100 secretion, with no significant added effect of ezetimibe.29 Taken together, it is possible that ezetimibe increased cholesterol efflux from

![Figure 2. Effects of ezetimibe on fecal excretion of total (A), endogenous (B), and dietary cholesterol (C), and relative contributions to fecal excretion of total neutral sterols by ezetimibe (D). Fecal excretion of total and endogenous cholesterol was determined in subjects receiving 10 mg/d ezetimibe (n=12) or placebo (n=12) for 6 weeks, as described in Methods. Fecal excretion of unabsorbed dietary cholesterol and unlabeled cholesterol was calculated as described in Methods. Results of A, B, and C are expressed as percent change relative to pretreatment in each group. Treatment effects and the P values are shown above the bars of placebo and ezetimibe. Results of D are increases of fecal excretion of endogenous cholesterol (FEEC), of dietary cholesterol excretion (FEDC), and of unlabeled cholesterol (FEUC), as percentage of the mean increase in fecal excretion of total neutral sterols by ezetimibe.](image-url)
Ezetimibe treatment increased the overall efficiency of the RCT pathway. In addition to inhibiting intestinal cholesterol absorption and reducing LDL cholesterol, ezetimibe increased FEEC without affecting the size of the rapidly mixing cholesterol pool, leading to increased percent endogenous cholesterol excretion. The ability of ezetimibe to increase RCT was quantitatively much larger than the effect on LDL cholesterol. Additional clinical investigation is needed to determine whether ezetimibe-enhanced RCT adds clinical benefit to LDL reduction.

Acknowledgments
We thank the Alvin J. Siteman, Cancer Center at Washington University School of Medicine for use of the Biologic Therapy Core Facility. We are grateful for the skilled assistance from the Center for Clinical Studies, the Clinical Research Unit nursing staff and the metabolic kitchen staff at Washington University Institute of Clinical and Translational Sciences. We appreciate the dedication of the study participants.

Sources of Funding
This work was supported by National Institutes of Health (NIH) grant R01 HL108160, the Washington University Mass Spectrometry Resource NIH/National Institute of General Medical Sciences grant P41GM103422, the Washington University Diabetes Research Center National Institute of Diabetes and Digestive and Kidney Diseases grant P30 DK020579, and the Washington University Nutrition Obesity Research Center NIH grant P30 DK056341. Research reported in this publication also was supported by the Washington University Institute of Clinical and Translational Sciences grant UL1 TR000448 from the National Center for Advancing Translation Sciences (NCATS) of the NIH. The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH.

Disclosures
Ezetimibe and placebo pills were provided by Merck (Kenilworth, NJ).

References


**Highlights**

- Ezetimibe (10 mg/d for 6 weeks) significantly increased fecal excretion of total neutral sterols 54.0±11.0% (P=0.0004), endogenous cholesterol 66.6±12.2% (P<0.0001), and unabsorbed dietary cholesterol 52.0±8.5% (P<0.0001).
- Ezetimibe did not alter fecal excretion of bile acids.
- Endogenous cholesterol excretion accounted for 86.6±11.9% of the increase in fecal neutral steroids in response to ezetimibe.
- Ezetimibe did not alter the size of the rapidly mixing cholesterol pool, but increased its turnover, as indicated by a 26.2±3.6% decrease in plasma cholesterol d7 enrichment (P=0.0096).
- Ezetimibe increased the efficiency of whole body cholesterol excretion (ie, greater cholesterol flux out of the rapid cholesterol pool).
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Arterioscler Thromb Vasc Biol. 2017;37:990-996; originally published online March 9, 2017; doi: 10.1161/ATVBAHA.117.309119
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Individual fecal excretion of endogenous cholesterol
Fecal excretion of endogenous cholesterol was measured as described in Materials and Methods in placebo (n=12) or ezetimibe (n=12) groups before and after treatment for 6 weeks.
Materials and Methods

Subjects and Eligibility Criteria
Females and males aged 30-80 years, in good health, with LDL cholesterol between 2.59 and 5.17 mmol/L (100-200 mg/dL), triglycerides < 4.52 mmol/L (400 mg/dL), hemoglobin A1c < 6.5%, and body mass index < 40.0 kg/m² were invited to participate. Individuals were excluded if they were pregnant or breastfeeding; taking statins, ezetimibe, niacin, plant sterols, bile acid sequestrants or drugs affecting lipid metabolism; had diabetes, gastrointestinal or liver disease; or had dietary restrictions that precluded adhering to our metabolic diet. All subjects provided written informed consent. The study was approved by the Washington University Institutional Review Board and was carried out at Washington University School of Medicine from February 2014 to May 2015.

Stable Isotope Tracers
[25,26,26,26,27,27,27]2H7-cholesterol was purchased from Sigma; [2,2,4,4,6]2H5-cholesterol and [5,6,22,23]3H4-sitostanol were obtained from Medical Isotopes. Intravenous infusates containing approximately 38 mg of cholesterol-d7 per mL were produced using current Good Manufacturing Practices in the Biologic Therapy Core Facility of Washington University School of Medicine (FDA facility establishment identifier 3007743644) and stored there under controlled conditions at 4°C for up to 9 months until use. Oral tracers were completely dissolved in olive oil by warming at 37°C to assure bioavailability; 100 mg of solubilized material containing 0.5 mg cholesterol-d5 and 0.5 mg sitostanol-d4 was added to each gelatin capsule.

Ezetimibe Treatment
After the pre-treatment cholesterol metabolism measurement, subjects were randomized to either ezetimibe or placebo for 6 weeks. Ezetimibe and identical-looking placebo tablets were provided by Merck. Treatment was double-masked, with neither the subject nor the study team knowing the treatment assignment until sample analyses were completed.

Cholesterol- and Phytosterol-Controlled Diet
To enable quantification of cholesterol metabolism, subjects received a controlled diet prepared in the Washington University Center for Applied Research Sciences metabolic kitchen for 5 days during each cholesterol metabolic measurement period. The diet contained 200 mg cholesterol and 150 mg phytosterols per 2000 Kcal, with 30% of calories as fat (7% saturated fat). The energy level was individualized to be weight-maintaining. All foods and caloric beverages were provided in coolers and subjects were instructed not to consume additional energy-containing items.

Protocol
As shown in Figure 1, the 8-week protocol included a 2-week pre-treatment cholesterol metabolic measurement period, a 6-week treatment period with ezetimibe or placebo tablets, and a post-treatment cholesterol metabolic measurement during the final 2 weeks of the treatment period.

On days 1 and 43, subjects received cholesterol-d7 in about 10 mL Intralipid® intravenously over 20 minutes. During days 10-14 and days 52-56, subjects consumed the sterol-controlled diet and took oral tracer capsules twice daily. Stool samples were collected on the last 2 days of each metabolic diet (days 13-14 and 55-56). Fasting blood samples were collected 4 times during the pre-treatment cholesterol metabolism measurement period (days 1, 2, 12, and 15) and 4 times during the post-treatment period (days 43, 44, 54, and 57).
Plasma sample analyses
Plasma phytosterols (campesterol, sitosterol, and stigmasterol), 5α-cholestanol, and lathosterol were measured using Gas Chromatography (GC) / tandem Mass Spectrometry (MS) on a Thermo TSQ8000. The parent-transition ions for d0-campesterol (d5-campesterol), d0-stigmasterol (d5-stigmasterol), d0-sitosterol (d5-sitosterol), d0-5α-cholestanol (d5-5α-cholestanol), and d0-lathosterol (d5-lathosterol) were 382.4-367.4 (386.4-371.4), 394.4-255.3 (398.4-259.2), 396.5-381.5 (400.4-259.3), 430.6-215.1 (435.4-219.2), and 428.5-213.4 (432.5-233.2), respectively. These values were normalized by expressing them relative to plasma total cholesterol concentration.

Total cholesterol and glycerol-blanked triglycerides were measured by automated enzymatic commercial kits. HDL (Roche Diagnostics USA, Indianapolis, IN) and LDL cholesterol (Sekisui, Lexington, MA, USA) were measured with direct assay kits.

Stool sample analyses
Aliquots of stool samples were saponified, extracted, and analyzed for cholesterol, coprostanol, coprostanone, and sitostanol using GC / electron ionization MS, with 5α-cholestane and hyodeoxycholic acid as internal standards. The results of the two stool collections were averaged.

Fecal bile acids were converted to n-butanol esters and trimethylsilyl ethers and analyzed by GC/MS. Twenty µg each of hyodeoxycholic acid and 5α-cholestanol were added as internal standards to 10–15 mg freeze-dried stool. The contents were dried and 200 µL of n-butanol and 50 µL concentrated hydrochloric acid were added, then butyl ester formation was performed by heating at 60°C for 4 hours. The esterified product was directly subjected to trimethylsilylation by adding 100 µL of Acetonitrile:BSTFA:Pyridine at 1:3:1 for 1 hour at 65°C. BSTFA [Bis(Trimethylsilyl) Trifluoro-Acetamide and 10% Trimethylchlorosilane] was from REGIS® Technologies, Inc. cat. #: 270131. Solvents were evaporated at 55°C under N2 and the trimethylsilyl ether derivatives formed were taken in 200 µL of hexane, centrifuged to separate the stool debris, and 1 µL of the clear supernatant was injected into a RTX-200MS GC column (Restek, 0.25 mmID, 0.5 µm df, cat. # 15638).

For quantification, the bile acids were monitored with ions m/z 414.3 (liothocholic acid and isolithocholic acid), m/z 412.3 (deoxycholic acid, isodeoxycholic acid, chenodeoxycholic acid, hyodeoxycholic acid), m/z 410.3 (cholic acid), m/z 502.3 (ursodeoxycholic acid), and m/z 428.3 (7-keolithocholic acid). The sterols were monitored with ions m/z 370.3 (coprostanol), m/z 403.3 (5α-cholestanol), m/z 369.3 (cholesterol), m/z 373.5 (d5-cholesterol), m/z 375.5 (d7-cholesterol), m/z 473.3 (sitostanol), m/z 477.3 (d4-sitostanol), m/z 372.3 (5α-cholestanol), and m/z 386.3 (coprostanone).

Calculations
Percent cholesterol absorption, fecal total neutral sterol excretion, and excretion of cholesterol metabolites were calculated as described previously. Fecal excretion of total neutral sterols and metabolites was corrected for fecal recovery of the nonabsorbable marker [2H4]sitostanol. Fecal excretion of total neutral sterols (g/day) was calculated as [(cholesterol + coprostanol + coprostanone)_{feces} g / g [2H4]sitostanol_{feces}] x [2H4]sitostanol g/day. Percent cholesterol absorption was calculated as 100 x [1
(([^2]H_{3})cholesterol_{feces}/[^2]H_{4}sitostanol_{feces})/([^{2}H_{5}]cholesterol_{capsule}/[^2]H_{4}sitostanol_{capsule})].
Fecal excretion of total neutral sterols is comprised of material of endogenous origin labeled with cholesterol-d7, of dietary origin labeled with cholesterol-d5, and unlabeled cholesterol. Fecal excretion of endogenous cholesterol was calculated as fecal excretion of total neutral sterols (g/day) x ([2H7]cholesterol_feces / [2H7]cholesterol_plasma of previous day). Fecal excretion of dietary cholesterol was calculated as dietary cholesterol intake multiplied by (100-% cholesterol absorption)/100. Fecal excretion of unlabeled cholesterol represents newly-synthesized hepatobiliary cholesterol that has not equilibrated with the tracers and was calculated by subtracting fecal cholesterol of endogenous origin and fecal cholesterol of dietary origin from total fecal neutral sterols. The relative contributions of the three sources of fecal cholesterol (i.e., endogenous cholesterol, dietary cholesterol, and unlabeled cholesterol) in response to ezetimibe treatment were computed as percentages of the mean increase in fecal excretion of total neutral sterols by ezetimibe. The amount of fecal bile acids in the analyzed sample was calculated from recovery of hyodeoxycholic acid internal assay standard and converted to grams of bile acids / day by determining the ratio of 5α-cholestane to the oral fecal flow marker sitostanol-d4. The formula for calculating fecal bile acids was [(bile acids_feces g / g 5α-cholestane) x (5α-cholestane / [2H4]sitostanol_feces x [2H4]sitostanol g/day).

The size of the rapidly-mixing cholesterol pool was calculated as the amount of cholesterol-d7 infused divided by the net increase in plasma cholesterol-d7 enrichment 24 hours later. Percent cholesterol excretion was expressed as the percent of the rapidly-mixing cholesterol pool excreted per day in the feces. The plasma relative cholesterol d7 enrichment for the pre-treatment period was computed as the ratio (plasma cholesterol d7 enrichment on day 15 – plasma cholesterol d7 enrichment on day 1) / (plasma cholesterol d7 enrichment on day 2 – plasma cholesterol d7 enrichment on day 1). The plasma relative cholesterol d7 enrichment for post-treatment was computed as the ratio (plasma cholesterol d7 enrichment on day 57 - plasma cholesterol d7 enrichment on day 43) / (Plasma cholesterol d7 enrichment on day 44 – plasma cholesterol d7 enrichment on day 43).

Statistical Analyses
To determine the effects of ezetimibe treatment versus placebo, repeated measures mixed random effects models were used to analyze changes over time by treatment group. Subject within treatment was a random effect and accounted for the correlation between pre- and post-treatment time points. Three contrasts, change with placebo, change with ezetimibe treatment, and difference in change between treatments were tested. Differences in variance between treatments were modeled with covariation parameters as needed. Changes in fecal excretion variables were analyzed with two-sample t-tests. To determine the effects of ezetimibe treatment on the three sources of fecal cholesterol excretion, mixed random effects ANOVA was used, with subject as a random effect. Relative turnover of cholesterol was assessed with a two-sample t-test. All analyses were performed using SAS version 9.4.
References


Overview of study protocol
Abbreviations: IV, intravenous infusion; Chol-d<sub>d<sub>7</sub></sub>, cholesterol-d<sub>d<sub>7</sub></sub>; Sit-d<sub>4</sub>, sitostanol-d<sub>4</sub>. **: Fasting blood was drawn on days 1, 2, 12, 15, 43, 44, 54 and 57.