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/m^2 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]$^2H_7$-cholesterol was purchased from Sigma; [2,2,4,4,6]$^2H_5$-cholesterol and [5,6,22,23]$^2H_4$-sitostanol were obtained from Medical Isotopes. Intravenous infusates containing approximately 38 mg of cholesterol-d_7 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 4C for up to 9 months until use.[1] Oral tracers were completely dissolved in olive oil by warming at 37C to assure bioavailability; 100 mg of solubilized material containing 0.5 mg cholesterol-d_5 and 0.5 mg sitostanol-d_4 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-d_7 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 d_0-campesterol (d_5-campesterol), d_0-stigmasterol (d_5-stigmasterol), d_0-sitosterol (d_5-sitosterol), d_0-5α-cholestanol (d_5-5α-cholestanol), and d_0-lathosterol (d_4-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.[2] 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.[3] Twenty µg each of hyodeoxycholic acid and 5α-cholestane 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 60C 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 N_2 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 (lithocholic acid and isolithocholic acid), m/z 412.3 (deoxycholic acid, isodeoxycholic acid, chenodexycholic 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 (d_5-cholesterol), m/z 375.5 (d_7-cholesterol), m/z 473.3 (sitostanol), m/z 477.3 (d_4-sitostanol), m/z 372.3 (5α-cholestane), 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.[4] Fecal excretion of total neutral sterols and metabolites was corrected for fecal recovery of the nonabsorbable marker $[^2H_4]$sitostanol. Fecal excretion of total neutral sterols (g/day) was calculated as $[(\text{cholesterol + coprostanol + coprostanone})_{\text{feces}}\ \text{g} / \text{g}\ [^2H_4]\text{sitostanol}_{\text{feces}})] \times [^2H_4]$sitostanol g/day. Percent cholesterol absorption was calculated as $100 \times [1$ $([_2H_5]\text{cholesterol}_{\text{feces}}/[^2H_4]\text{sitostanol}_{\text{feces}})/([^2H_5]\text{cholesterol}_{\text{capsule}}/[^2H_4]\text{sitostanol}_{\text{capsule}})]$.

Fecal excretion of total neutral sterols is comprised of material of endogenous origin labeled with cholesterol-d_7, of dietary origin labeled with cholesterol-d_5, and unlabeled cholesterol. Fecal excretion of endogenous cholesterol was calculated as fecal excretion of total neutral sterols (g/day) x ($[^2H_7]$cholesterol$_{feces}$ / $[^2H_7]$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.[5] 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-d_4. The formula for calculating fecal bile acids was [(bile acids$_{feces}$ g / g 5α−cholestane) x (5α−cholestane / $[^2H_4]$sitostanol)$_{feces}$ x $[^2H_4]$sitostanol g/day.

The size of the rapidly-mixing cholesterol pool was calculated as the amount of cholesterol-d_7 infused divided by the net increase in plasma cholesterol-d_7 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 d_7 enrichment for the pre-treatment period was computed as the ratio (plasma cholesterol d_7 enrichment on day 15 – plasma cholesterol d_7 enrichment on day 1) / (plasma cholesterol d_7 enrichment on day 2 – plasma cholesterol d_7 enrichment on day1). The plasma relative cholesterol d_7 enrichment for post-treatment was computed as the ratio (plasma cholesterol d_7 enrichment on day 57 - plasma cholesterol d_7 enrichment on day 43) / (Plasma cholesterol d_7 enrichment on day 44 – plasma cholesterol d_7 enrichment on day 43).

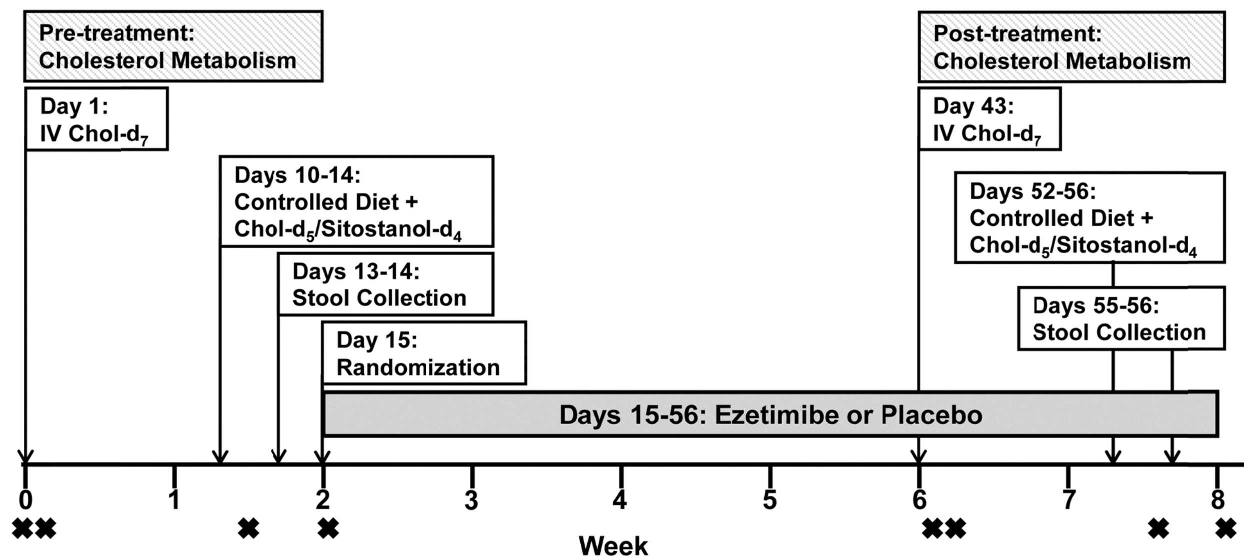
Statistical Analyses

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 covariance 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

1. Lin X, Ma L, Racette SB, Swaney WP, Ostlund RE, Jr. Preparation of intravenous cholesterol tracer using current good manufacturing practices. *J Lipid Res*. 2015;56:2393-2398
2. Lin X, Racette SB, Lefevre M, Ma L, Spearie CA, Steger-May K, Ostlund RE, Jr. Combined effects of ezetimibe and phytosterols on cholesterol metabolism: A randomized, controlled feeding study in humans. *Circulation*. 2011;124:596-601
3. Batta AK, Salen G, Rapole KR, Batta M, Batta P, Alberts D, Earnest D. Highly simplified method for gas-liquid chromatographic quantitation of bile acids and sterols in human stool. *J Lipid Res*. 1999;40:1148-1154
4. Racette SB, Lin X, Lefevre M, Spearie CA, Most MM, Ma L, Ostlund RE, Jr. Dose effects of dietary phytosterols on cholesterol metabolism: A controlled feeding study. *Am J Clin Nutr*. 2010;91:32-38
5. Ferezou J, Coste T, Chevallier F. Origins of neutral sterols in human feces studied by stable isotope labeling (d and 13c). Existence of an external secretion of cholesterol. *Digestion*. 1981;21:232-243

Figure 1



Overview of study protocol
Abbreviations: IV, intravenous infusion; Chol-d_5/d_7, cholesterol-d_5/d_7; Sit-d_4, sitostanol-d_4. ✖ : Fasting blood was drawn on days 1, 2, 12, 15, 43, 44, 54 and 57.