Materials and Methods

Research subjects and eligibility criteria

Healthy subjects and individuals with medical conditions stable for at least 6 months, comprising all races and both genders, and aged 30-80 years were invited to participate. Exclusion criteria were current smoking, pregnancy, breast feeding, liver disease, gastrointestinal and renal disorders, hemoglobin A1c (HbA1c) $\geq$ 6.5%, LDL cholesterol > 190 mg/dL, fasting triglycerides > 400 mg/dL, or consumption of phytosterol supplements, bile acid sequestrants, ezetimibe, niacin, or other non-statin antilipidemic drugs. Oral contraceptives and common medications for stable medical conditions, including statin drugs, were allowed. The study was approved by The Institutional Review Board of Washington University in St. Louis and written informed consent was obtained from each subject.

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 of 10 mL Intralipid® containing about 30 mg cholesterol-d_7 were prepared using Current Good Manufacturing Practices in the Biologic Therapy Core Facility of Washington University (FDA facility establishment identifier 3007743644) and stored under controlled conditions at 2-8C for up to 9 months before use.[1] To ensure bioavailability, solid oral tracers were completely dissolved in olive oil by warming at 37C; 100 mg of this solution was then added to a gelatin capsule that contained 0.5 mg cholesterol-d_5 (an oral cholesterol tracer) and 0.5 mg sitostanol-d_4 (a nonabsorbable fecal marker). Capsules were stored at room temperature. Subjects were instructed to consume one capsule twice daily with meals during the 5-day controlled diet period.

Cholesterol- and phytosterol-controlled diet

To enable quantification of cholesterol excretion, subjects received a controlled diet prepared in the metabolic research kitchen of the Washington University in St. Louis ICTS Clinical Research Unit for the last 5 days of the measurement period. The diet contained 200 mg cholesterol and 150 mg phytosterols per 2000 Kcal, with 30% of calories as fat (7% as saturated fat). The energy level was individualized to be weight-maintaining. All foods and caloric beverages were provided and subjects were instructed not to consume additional energy-containing items.

Clinical protocol

During a screening visit, demographic data, medical history and medications were recorded, blood pressure was measured and blood was drawn for lipid, chemistry and blood count panels. Eligible subjects then underwent a 2-week clinical protocol (Figure 1). The rapidly-mixing endogenous cholesterol pool was labeled using a 20-minute intravenous infusion of 30 mg cholesterol-d_7 on day 1; plasma samples were drawn on days 1, 2, 12, and 15 to determine plasma cholesterol-d_7 enrichment. The rapidly-mixing cholesterol pool size was determined from the dilution of tracer in plasma on day 2. In order to improve data reproducibility and precision, the cholesterol metabolic measurements were made on days 13-14, which allowed partial equilibration of the

tracer with endogenous cholesterol pools and avoided rapid changes in plasma cholesterol enrichment. Beginning at breakfast on day 10 and ending before breakfast on day 15, subjects consumed the sterol-controlled diet and took oral tracer capsules twice daily with meals. Fecal samples were collected on days 13 and14.

Plasma sample analyses

Total cholesterol, triglycerides and direct HDL cholesterol were measured on a Roche Diagnostics Cobas 6000 autoanalyzer. LDL cholesterol was calculated by the Friedewald equation. Plasma 5α-cholestanol (a proposed biomarker for cholesterol absorption) and lathosterol (a proposed biomarker for cholesterol biosynthesis) were measured using gas chromatography/tandem mass spectrometry with a Thermo TSQ8000 triple quadrupole instrument. Ion transitions for 5α-cholestanol-d_0, 5α-cholestanol-d_5, lathosterol-d_0, and lathosterol-d_4 were 430.6-215.1, 435.4-219.2, 428.5-213.4, and 432.5-233.2, respectively. Cholesterol-d_7 enrichment in plasma also was measured using GC/MS with ion transitions of 368.4-353.4 and 375.4-255.4, for cholesterol-d_0 and cholesterol-d_7, respectively.

Stool sample analyses

Stool samples from each subject were mixed thoroughly to ensure uniformity. Aliquots of stool samples were saponified, extracted, and analyzed for cholesterol, coprostanol, coprostanone, bile acids, and sitostanol using sitostanol-d_4 as a fecal flow marker and 5α-cholestane and hyodeoxycholic acid as internal assay standards.[2] The ion transitions for cholesterol-d_0, cholesterol-d_5, cholesterol-d_7, coprostanol, coprostanone, sitostanol-d_0, and sitostanol-d_4 were 368.-353.4, 372.4-247.1, 375.4-360.4, 370.4-215.2, 316.4-161.2, 398.4-257.3, and 402.5-259.4, respectively. 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-d_0), m/z 373.5 (cholesterol-d_5), m/z 375.5 (cholesterol-d_7), m/z 473.3

(sitostanol-d_0), m/z 477.3 (sitostanol-d_4), 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 [(cholesterol + coprostanol + coprostanone)$_{feces}$ g / g [$^2H_4$]sitostanol$_{feces}$)] x [$^2H_4$]sitostanol g/day. Percent cholesterol absorption was calculated as 100 × [1 - ([$_2H_5$]cholesterol$_{feces}$/[$^2H_4$]sitostanol$_{feces}$)/([$^2H_5$]cholesterol$_{capsule}$/[$^2H_4$]sitostanol$_{capsule}$)].

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 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 cholesterol d_7 enrichment change from day 15 to day 2 was computed as (plasma cholesterol d_7 enrichment on day 2 - plasma cholesterol d_7 enrichment on day 15) / plasma cholesterol d_7 on day 2 * 100.

CIMT measurements

CIMT was measured with a 7-MHz linear-array transducer by trained sonographers following a standardized protocol. An experienced cardiologist, masked to subject characteristics, read and interpreted the results in a core laboratory as described previously.[6-9] B-mode images of both carotid arteries were obtained in the longitudinal axis, with careful attention to ensure that the distal 1 cm region of the common carotid artery just proximal to the bifurcation was well defined. CIMT was calculated from end-diastolic B-mode images as the average of two 1-cm segments in the distal wall from both right and left common carotid arteries, excluding raised lesions and plaques when present, measured using an automated system with an edge detection algorithm and

manual override capacity. One hundred separate dimensional measurements were obtained from the 1-cm segment and averaged to obtain mean CIMT.

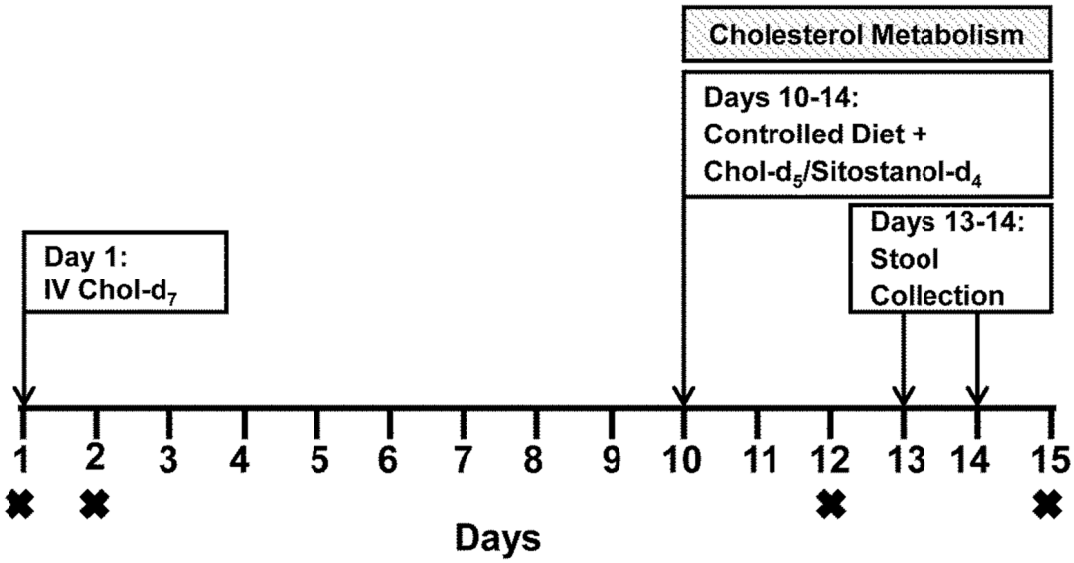
Statistics

Mean and standard deviation of each outcome variable were computed to describe subject characteristics. Relationships between variables were estimated and tested with Pearson correlation and simple linear regression analyses. Multiple regression was used to estimate associations of CIMT on FEEC, while controlling for age, gender, systolic blood pressure, HbA1c, LDL cholesterol, HDL cholesterol, statin drug use, and the ratio of 5α-cholestanol/lathosterol. All analyses were performed with SAS 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
6. de las Fuentes L, Gu CC, Mathews SJ, Reagan JL, Ruthmann NP, Waggoner AD, Lai CF, Towler DA, Davila-Roman VG. Osteopontin promoter polymorphism is associated with increased carotid intima-media thickness. *J Am Soc Echocardiogr*. 2008;21:954-960
7. de las Fuentes L, Waggoner AD, Mohammed BS, Stein RI, Miller BV, 3rd, Foster GD, Wyatt HR, Klein S, Davila-Roman VG. Effect of moderate diet-induced weight loss and weight regain on cardiovascular structure and function. *Journal of the American College of Cardiology*. 2009;54:2376-2381
8. Painschab MS, Davila-Roman VG, Gilman RH, Vasquez-Villar AD, Pollard SL, Wise RA, Miranda JJ, Checkley W, Group CCS. Chronic exposure to biomass fuel is associated with increased carotid artery intima-media thickness and a higher prevalence of atherosclerotic plaque. *Heart*. 2013;99:984-991
9. Fleg JL, Mete M, Howard BV, Umans JG, Roman MJ, Ratner RE, Silverman A, Galloway JM, Henderson JA, Weir MR, Wilson C, Stylianou M, Howard WJ. Effect of statins alone versus statins plus ezetimibe on carotid atherosclerosis in type 2 diabetes: The sands (stop atherosclerosis in native diabetics study) trial. *Journal of the American College of Cardiology*. 2008;52:2198-2205

Figure 1



Timeline of study protocol
Subjects received intravenous cholesterol-d_7 on day 1. Cholesterol metabolism was measured from days 10 to 15 while the subjects were on a cholesterol- and phytosterol-controlled diet. Oral tracers of oil-solubilized cholesterol-d_5 and sitostanol-d_4 were administered in 2 capsules a day for five days with stool collected on days 13 and 14. Fasting blood samples were drawn on days marked by X.