Measurement of Intestinal and Peripheral Cholesterol Fluxes by a Dual-Tracer Balance Method

Ronda, Onne A H O; van Dijk, Theo H; Verkade, H J; Groen, Albert K

Published in:
Current protocols in mouse biology

DOI:
10.1002/cpmo.16

Publication date:
2016

Measurement of Intestinal and Peripheral Cholesterol Fluxes by a Dual-Tracer Balance Method

Onne A.H.O. Ronda,[1] Theo H. van Dijk,[2] H.J. Verkade,[1] and Albert K. Groen[2,3]

[1]Center for Liver, Digestive and Metabolic Diseases, Departments of Pediatrics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[2]Department of Laboratory Medicine, Center for Liver Digestive and Metabolic Diseases, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[3]Amsterdam Diabetes Center, Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands

Long-term elevated plasma cholesterol levels put individuals at risk for developing atherosclerosis. Plasma cholesterol levels are determined by the balance between cholesterol input and output fluxes. Here we describe in detail the methodology to determine the different cholesterol fluxes in mice. The percentage of absorbed cholesterol is calculated from a stable isotope–based double-label method. Cholesterol synthesis is calculated from MIDA after ^{13}C-acetate enrichment. Cholesterol is removed from the body via the feces. The fecal excretion route is either biliary or non-biliary. The non-biliary route is dominated by trans-intestinal cholesterol efflux, or TICE. Biliary excretion of cholesterol is measured by collecting bile. Non-biliary excretion is calculated by computational modeling. In this article, we describe methods and procedures to measure and calculate dietary intake of cholesterol, fractional cholesterol absorption, fecal neutral sterol output, biliary cholesterol excretion, TICE, cholesterol synthesis, peripheral fluxes, and whole-body cholesterol balance.

Keywords: cholesterol absorption • cholesterol synthesis • biliary excretion • fecal cholesterol excretion • mouse • trans-intestinal cholesterol excretion

How to cite this article:
Ronda, O.A.H.O., van Dijk, T.H., Verkade, H.J. and Groen, A.K. 2016. Measurement of intestinal and peripheral cholesterol fluxes by a dual-tracer balance method. *Curr. Protoc. Mouse Biol.* 6:408-434. doi: 10.1002/cpmo.16

INTRODUCTION

Cholesterol is a central lipid in mammalian cells. It is a crucial constituent of cell membranes (Ikonen, 2008), and is used for the synthesis of steroid hormones, vitamin D, and bile acids (van der Velde et al., 2010; Temel and Brown, 2012). An excess of cholesterol in blood is the primary risk factor for cardiovascular disease in humans. Almost all body cells are able to synthesize cholesterol (Ikonen, 2008). In these cells, the metabolic system strives to maintain a steady-state concentration of cholesterol, partly by movement of cholesterol and partly by de novo synthesis. Humans and rodents cannot catabolize sterols in their cells or in their gastrointestinal tract (Kudchodkar et al., 1972). Therefore, removal of cholesterol from the body takes place by fecal excretion in the form of acidic (bile acids) or neutral steroids. An excess of cholesterol in the periphery is resolved by reverse cholesterol transport (RCT). RCT is defined as cholesterol flux

Current Protocols in Mouse Biology 6:408-434, December 2016
Published online December 2016 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpmo.16

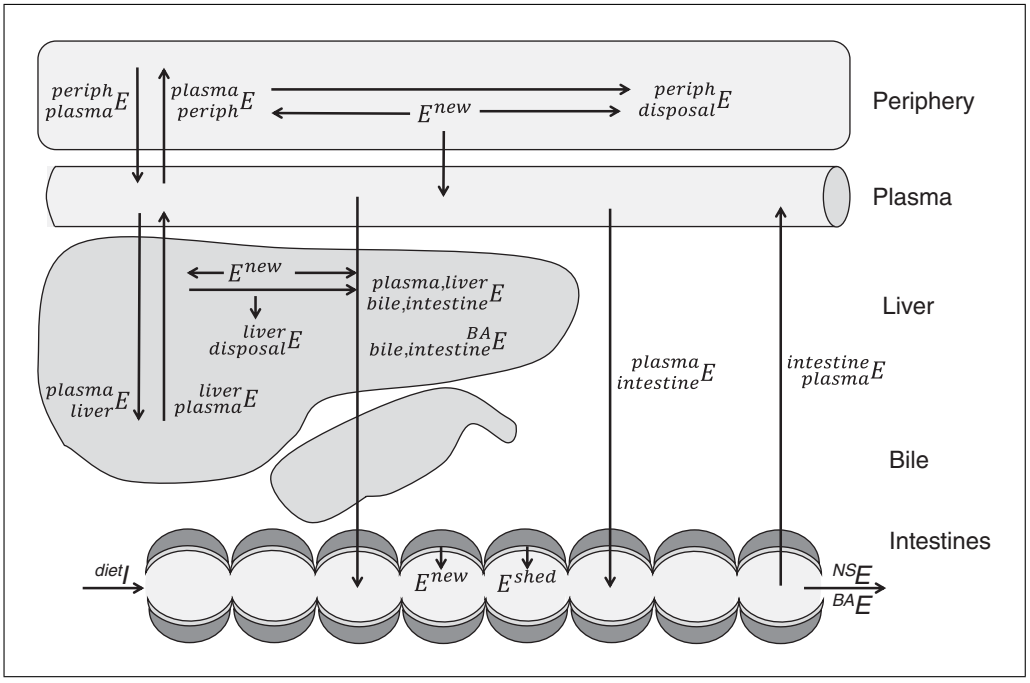

Figure 1 Schematic representation of the working model, showing the major cholesterol fluxes that are measured, calculated, or discussed in the protocol. BA: bile acid, NS: neutral sterol.

from the periphery via the liver to the feces (Fielding and Fielding, 1995; Groen et al., 2004; Heinecke, 2012). In the past, it was thought that cholesterol was removed from the body via hepatobiliary secretion. Now it has become clear that cholesterol can also be secreted directly from blood into the intestinal lumen. This pathway has become known as trans-intestinal cholesterol efflux or TICE (van der Velde et al., 2007; van der Velde et al., 2010; Brufau et al., 2011; reviewed in Temel and Brown, 2012). In mice, approximately one-third of all fecal neutral sterol excretion is directly caused by TICE, which can be increased to approximately two-thirds by pharmacologically stimulating the Liver X Receptor (LXR; van der Veen et al., 2009), indicating the capability and flexibility of TICE. The enterocytes in the intestinal wall can also produce cholesterol de novo and directly secrete it into the lumen (van der Veen et al., 2009; Brufau et al., 2011). In addition, intestinal shedding causes cholesterol to enter the lumen. This means that a fraction of intestinal-lumen cholesterol does not originate from the plasma. In this article, we describe four methods, namely: a method for calculating dietary cholesterol intake and neutral sterol and bile acid excretion (Basic Protocol 1), a method for calculating cholesterol absorption by dual-tracer techniques (Basic Protocol 2), a method for calculating cholesterol synthesis (Basic Protocol 3), and a method for calculating biliary and non-biliary cholesterol excretion (Basic Protocol 4).

Working Model

Before commencing the description and the execution of the animal studies, it is necessary to describe the kinetic model as utilized in this protocol. The kinetic model (Fig. 1) used in this protocol covers peripheral tissue, plasma, liver, bile, and intestine. Peripheral tissue includes all tissue inside the system (the animal) except for plasma, liver, bile, and intestines. In the model, it is assumed that peripheral cholesterol flux occurs to (${}^{periph}_{plasma}E$) and from (${}^{plasma}_{periphery}E$) plasma. If the cycling duration of a tracer exceeds the timespan of the experiment, the cholesterol is said to be 'disposed' (${}^{periph}_{disposal}E$). Cholesterol synthesis takes place in the periphery. The plasma serves as a pool that cannot synthesize cholesterol itself. Cholesterol flux takes place from plasma into the liver (${}^{plasma}_{liver}E$), which is partially excreted into the intestine, without (${}^{liver}_{bile,intestines}E$) or with conversions

into bile salts (${}^{Bileacids}_{bile,intestines}E$). Both fluxes are mediated via the bile. Cholesterol can be stored in the liver (${}^{liver}_{disposal}E$) for the timespan of the experiment or recycled to plasma (${}^{liver}_{plasma}E$). The liver is capable of synthesizing new cholesterol. Dietary cholesterol enters the intestinal lumen (${}^{diet}I$). The intestines exchange cholesterol to the plasma via absorption (${}^{intestines}_{plasma}E$) and from the plasma via TICE (${}^{plasma}_{intestines}E$). The intestines synthesize cholesterol de novo, and the intestinal lining is continuously shed (E^{shed}). A fraction of luminal cholesterol which is partially converted into neutral sterols (${}^{NS}E$), and bile acids (${}^{BA}E$), is excreted from the system into the feces. The total body cholesterol synthesis (E^{new}) can be calculated. In addition, it is possible to calculate an overall cholesterol balance (${}^{balance}E$)—the gastrointestinal sterol balance—which is an approximation of the whole-body sterol balance.

General Considerations for Designing an Animal Study

The ultimate design of an animal experiment largely depends on the research question and the specific need for information. In typical experiments designed to assess intestinal cholesterol fluxes and cholesterol synthesis, we have been using a variety of techniques (Brufau and Groen, 2011; Freark de Boer et al., 2012; Grefhorst et al., 2012; Temel and Brown, 2012; van der Wulp et al., 2012a; Dikkers et al., 2014). These techniques can be combined into one coherent protocol that can be performed within the same cohort without causing severe discomfort to the animals. The individual techniques described are: dietary cholesterol intake, fecal neutral sterol (NS) output, cholesterol absorption, biliary excretion by bile cannulation, and cholesterol synthesis.

The protocols described here have been carefully designed and meticulously optimized for use in mice. However, by correcting for the difference in body weight, and taking into account that rats do not have a gall bladder, all of the described protocols can be performed in rats (Nibbering et al., 2001). In addition, we speculate that other mammalian (rodent) species can theoretically be used with minimal modification of the techniques. The use of stable isotope tracers (instead of radioactive tracers) enables the safe usage of some of the described techniques (Basic Protocols 1 and 2) in human (pediatric) subjects. We have validated an adapted form of the cholesterol absorption technique (Basic Protocol 2) in humans (Jakulj et al., 2013).

The calculation of dietary cholesterol intake is dependent on the quantification of food intake over a period of time. Accurate measurements are obtained when chow is pelleted and non-crumbling. When needed, chow crumbs can be manually recovered from the bedding. It is advised to measure food consumption per animal on a daily basis throughout the experiment. This allows for a cumulative count of all ingested cholesterol. The calculation of fecal neutral sterol output is dependent on the quantification of feces over a certain period. In many research institutes, it is no longer ethically permitted to use mesh wire floor cages due to the associated stress and discomfort. Alternatively, animals are individually housed in regular cages with sparse bedding, and the cage bedding is changed upon each sampling procedure. Feces are collected from the bedding by manual sorting. It is advised to sample feces a minimum of three times per animal throughout the experiment. However, it is preferable to sample feces on a daily basis throughout the experiment to allow for cumulative assessment of all excreted neutral sterols and bile acids. The calculation of cholesterol absorption is dependent on the measurement of dietary cholesterol intake, fecal NS output, D_5/D_7-cholesterol administration, and blood sampling. A blood spot, sampled from the tail tip and collected on sample carrier (filter) paper, is sufficient for modern GC/MS analysis. The calculation of peripheral fluxes and whole-body sterol balance is dependent on the calculation of cholesterol absorption and on biliary cholesterol excretion calculated by bile-duct cannulation. The calculation of

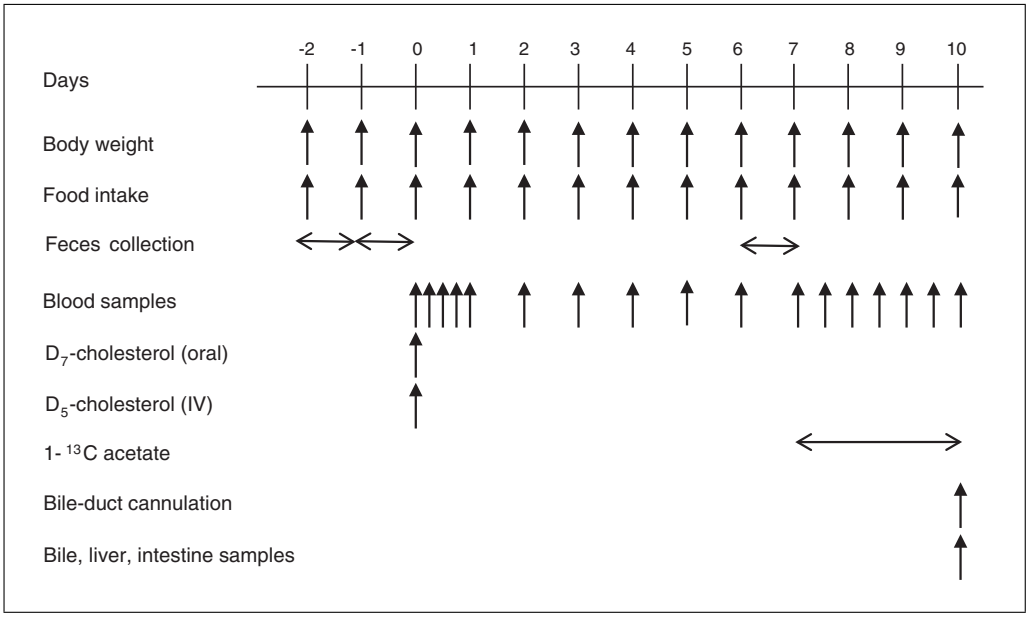

Figure 2 Experimental schedule for performing all protocols described in this paper within the same cohort. Day 0 to 7 is required for assessing cholesterol absorption. Day 7 to 10 is required for assessing cholesterol synthesis.

cholesterol synthesis as described within this paper is dependent on the calculation of cholesterol absorption and on labeled acetate administration.

The actual design per specific experiment is determined by the specific research question of the study. Each individual element of the kinetic characterization has its own requirements. The minimum measurement duration for plasma dual-tracer decay is 168 hr. The minimum time for measuring cholesterol synthesis is 72 hr. Preferably, cholesterol synthesis should be measured for 120 hr. Empirically, we have observed that the introduction of 1-^{13}C-acetate affected the plasma dual-tracer measurements, necessitating the segregation of cholesterol absorption and synthesis to separate periods in the experiment (Fig. 2). The measuring period of cholesterol absorption and synthesis should not overlap.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

DIETARY CHOLESTEROL INTAKE AND NEUTRAL STEROL AND BILE ACID EXCRETION

BASIC PROTOCOL 1

Cholesterol is absorbed from the gastrointestinal lumen by NPC1L1 (van der Veen et al., 2005). The percentage of cholesterol that is absorbed (fractional absorption) is calculated from dietary cholesterol. The fraction that is not absorbed (1 – fractional absorption) is partially metabolized by the microbiota into neutral sterols such as coprostanol and dihydrocholesterol (Kudchodkar et al., 1972; Dikkers et al., 2014). Neither humans nor their microbiota are capable of degrading the cyclopentanophenanthrene nucleus, which defines the sterol structure (Kudchodkar et al., 1972). This implies that sterols can only be removed by excretion. Rodents and their regular microbiota are similarly incapable of breaking down sterols. The microbiota of both humans and rodents are capable of metabolizing cholesterol. Collectively, cholesterol and its bacterial metabolites are called neutral sterols. Cholesterol is the parent molecule of bile acids, vitamin D, and steroid hormones. The relative contribution of vitamin D and steroid hormones is negligible for cholesterol fluxes and will not be included in the calculations. During the conversion of cholesterol to bile acids, the steroid nucleus remains preserved. One mole of

cholesterol is stoichiometrically related to the production of one mole of bile acids. Bile acids produced in the liver (the primary bile acids) are cholic acid and chenodeoxycholic acid (muricholic acid in mice) (Russell and Setchell, 1992). After conjugation with glycine or taurine, they are either temporarily stored in the gall bladder (humans, mice) or directly secreted into the intestinal lumen (rats). Fractional absorption of bile acids differs from absorption of cholesterol. Microbiota are capable of deconjugating bile acids (removal of glycine/taurine), and also of further metabolization such as 7-dehydroxylation, whereby hydrophobicity and absorption characteristics are strongly affected (Vrieze et al., 2014). Bile acids and cholesterol are not completely absorbed from the intestinal lumen. Consequently, a fraction of bile acids and cholesterol is disposed of via the feces and thereby lost from the system.

The system has a basic input of cholesterol via the diet and via de novo synthesis and an output of cholesterol via neutral sterols and bile acids in the feces. Calculating dietary cholesterol intake rate (^{diet}I) requires the measurement of food intake per animal per time unit and the cholesterol content in the diet. Likewise, calculating fecal neutral sterol excretion rate ($^{feces}E$) requires the quantification of feces output and the content of neutral sterols in the feces. Coprophagy (the consumption of feces) is (partly) avoidable when the animals are housed in wire-mesh-floor cages. The diameter of the wire-mesh floor is chosen based on the animal model; mouse droppings are typically 3 mm in diameter, whereas rat droppings are typically 6 mm in diameter. Coprophagy does not affect cholesterol fluxes per se. The cage containing an animal and its feces can be seen as a 'system', whereby feces are removed from the system (the cage) and diet is added. Indeed, a study performed in rats showed that coprophagy did not alter the hypocholesterolemic effects of oat bran (Jackson and Topping, 1993), indicating that coprophagy might have limited or no effect on cholesterol-related parameters. In some research institutes, using wire-mesh cages is no longer or has never been ethically permitted, necessitating individual housing in a conventional cage with a sparse amount of bedding.

Materials

Appropriate animal model (mouse, rat)
Rodent diet (pellets preferred over powder)
5α-cholestane (Sigma, cat. no. C8003, or equivalent)
1 M NaOH
Methanol
Petroleum ether, 60º to 80ºC boiling point
N_2 source
Coprostanol (Sigma, cat. no. C8003, or equivalent)
Epicoprostanol (Steraloids, cat. no. C5050, or equivalent)
Cholesterol (Sigma, cat. no. C8667, or equivalent)
Dehydrocholesterol (Sigma, cat. no. 30800)
Sitostanol (Stigmastanol, Sigma, cat. no. S4297)
N,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco, cat. no. 33027)
Pyridine
Trimethylchlorosilane (TMCS; Thermo, cat. no. TS-88530)
Hexane (heptane has similar properties and can be used interchangeably)
C18 Sep-Pak column (Waters)
10% (v/v) acetone
Isolithocholic acid (Steraloids, cat. no. C1475)
Lithocholic acid (Steraloids, cat. no. C1420)
α-Muricholic acid (Steraloids, cat. no. C1890)
β-Muricholic acid (Steraloids, cat. no. C1895)

ω-Muricholic acid (Steraloids, cat. no. C1888)
Deoxycholic acid (Sigma, cat. no. D2510)
Cholic acid (Sigma, cat. no. C1129)
Chenodeoxycholic acid (Sigma, cat. no. C9377)
Hyodeoxycholic acid (Steraloids, cat. no. C0860)
Ursodeoxycholic acid (Sigma, cat. no. U5127)
Hyocholic acid (Avanti Polar Lipids, cat. no. 700159)
5β-Cholanic acid-7α, 12α-diol (Steraloids, cat. no. C1170)
Acetyl chloride

Container to accommodate the animal during weighing
Individual housing (collection of feces)
Appropriate bedding [coarse wood chips, sparse paper-based nesting material; not preferred are cotton-based nestlets, (fine) sawdust, and (fine) wood shavings]
Scale with 0.1 g accuracy to weigh mice/rats
Scale with 1 mg accuracy to weigh food and feces
Mortar and pestle for grinding the diet and the feces
10-ml glass tubes that can be closed air-tight
50º, 55º, 65º, and 80ºC heat blocks or water baths
2-ml gas chromatography (GC) vials
Gas chromatograph

NOTE: The following steps can be applied to either mice or rats.

Obtain food and feces samples

1. On the day before the start of the experiment, weigh the food and transfer the animals to individual clean cages (also see Critical Parameters). Weigh the animals.

2. The following day, within the same part of the day (note down the exact time), collect the bedding (containing the feces and potentially chunks of the diet) and replace it with fresh bedding. Weigh the food remaining and meticulously include diet chunks present in the bedding. Separate the feces from the bedding.

   This procedure is performed for every day on which feces is collected.

3. Dry mouse (or rat) feces (preferably) by lyophilization (freeze-drying).

   Alternatively, feces can be air-dried by spreading it out on a paper surface and incubating for 48 hr at room temperature. After drying, feces can be stored in small ziplock bags, 2-ml microcentrifuge tubes, or other small plastic containers.

   Feces can be stored dry for several months in airtight plastic bags at room temperature.

4. Grind the feces and an aliquot of the food into a fine powder and homogenize.

   Dry feces generate static electricity when in contact with most types of plastic and will statically cling to surfaces.

5. Weigh 50 mg dried feces or food. Note the exact weight (to 0.1 mg accuracy). Include a known control sample. Transfer to a glass 10-ml tube that can be closed air-tight.

Extract neutral sterols from food and feces samples

6. Add 49 nmol 5α-cholestane (internal standard for neutral sterols, dissolved in 100 µl absolute ethanol). In addition, add 13 nmol of 5β-cholanic acid 7α,12α diol (internal standard for bile acids, dissolved in 100 µl absolute ethanol, not applicable for food samples).

7. Add 250 µl 1 M NaOH (dissolved in Milli-Q or deionized water) and 750 µl methanol. Close the tubes air-tight.

   CAUTION: *At any time when working with volatile organic compounds, work in the fume hood and wear protective gloves.*

8. Incubate the tubes at 80°C for 2 hr. Cool the tubes to room temperature afterwards.

9. Extract the neutral sterols; add 3 ml petroleum ether (60º to 80°C boiling point), vortex vigorously for 30 sec, and centrifuge 10 min at 900 × *g*, room temperature. Transfer the top petroleum layer to a clean glass tube.

10. Repeat the petroleum extraction described in step 9 on the non-transferred liquid two more times, transferring the petroleum ether into the same clean glass tube as the top layer each time, yielding a total of ~9 ml per sample in one clean glass tube.

11. Evaporate the sterol-containing petroleum ether under N_2 at 50°C.

    The bottom layer primarily consists of ethanol and methanol and a small portion of water, which contains the bile acids, and is stable when kept in the dark at room temperature. The bile acids are extracted and analyzed from step 17 onwards.

Prepare the neutral sterol standard curve

12. Prepare a standard curve by pipetting 0, 10, 25, 50, and 100 nmol of coprostanol, epicoprostanol, cholesterol, dihydrocholesterol, and sitostanol into the same tube, yielding a total of five standard solutions with different concentrations.

13. Add 49 nmol 5α-cholestane (internal standard for neutral sterols, can be dissolved in chloroform or benzene) to each calibration solution. Evaporate the solvent under N_2 at 50°C.

TMS derivatization

14. Perform a TMS derivatization on the isolated samples and the standard curve by adding 100 µl BSTFA:pyridine:TMCS (5:5:0.1).

15. Mix briefly and incubate for 1 hr at room temperature inside an air-tight tube.

16. Evaporate under N_2 at room temperature. Add 1 ml hexane, vortex vigorously, centrifuge 10 min at 900 × *g*, room temperature, and transfer the hexane fraction into a 2-ml GC vial.

    The reason for centrifugation is to ensure a complete transfer from one tube to the other without any phase-separation. Note that heptane has similar chemical properties to hexane and these solvents can be used interchangeably within this protocol.

    The sample is ready for GC analysis for determining neutral sterols. The parameters used for GC analysis are shown in Table 1.

    The concentration of each neutral sterol species can be calculated by performing linear regression. The ratio between the integrated neutral sterol peak and the integrated internal standard is calculated, after which the absolute amount can be calculated from the standard curve.

Extract bile acids from feces samples

17. The methanol fraction kept at step 11 contains the bile acids, which are dissolved by adding 9 ml of Milli-Q water or distilled, deionized water. Vortex vigorously.

    BA are not stable in water long-term and should preferably be extracted within one day. At any point within this article, Milli-Q water and distilled, deionized water can be interchanged.

18. Centrifuge 10 min at 900× *g*, room temperature. Hereby, fecal debris, which might interfere with the bile acid extraction, are centrifuged to the bottom of the tube.

19. Rinse a C18 Sep-Pak column with 4 ml methanol, 10 ml Milli-Q water, and 4 ml of 10% acetone in Milli-Q water, followed by 10 ml Milli-Q water.

20. Gently transfer the BA supernatant from a feces sample (step 18) or from calibration solution (from step 18) onto the column and rinse the column twice with 10 ml Milli-Q water.

21. Elute BA by placing 4 ml of 75% (v/v) methanol onto the column and gently flush the fluid into a clean 10-ml tube. Evaporate the eluate under N_2 at 65°C.

    Columns may be used to extract a second sample after a brief 10-ml rinse with Mill-Q water.

Prepare the bile acid standard curve

22. Prepare a standard curve by pipetting 0, 2.5, 5, 12.5, and 25 nmol of isolithocholic acid, lithocholic acid, α-muricholic acid, β-muricholic acid, ω-muricholic acid, deoxycholic acid, cholic acid, chenodeoxycholic acid, hyodeoxycholic acid, ursodeoxycholic acid, and hyocholic acid, yielding a total of five different standard solutions. Add 13.25 nmol internal standard (5β-cholanic acid-7α, 12α-diol) and evaporate the solvent under a stream of N_2 at 60°C.

23. Prepare 1:20 acetyl chloride:methanol by dropwise adding the acetyl chloride to the methanol while gently mixing (it is advisable to prepare 25% extra). Add 200 µl to each sample and standard.

    CAUTION: *When working with acetyl chloride, it is strongly advised to work with gloves in a fume hood.*

24. Close the tubes air-tight, mix, and incubate for 30 min at 55°C. Afterwards, evaporate under N_2 at 55°C.

25. Perform a TMS derivatization by adding 100 µl BSTFA:pyridine:TMCS (5:5:0.1).

26. Incubate for 1 hr at room temperature in an air-tight tube. Evaporate under N_2 at room temperature.

27. Add 1 ml hexane (alternatively, heptane can be used) and vortex vigorously. Centrifuge 10 min 900 × *g*, room temperature.

28. Transfer the whole fluid to a 2-ml GC vial. Evaporate and dissolve in 150 µl. Perform gas chromatography.

    Gas chromatography settings are given in Table 1. Each peak is integrated and the concentration of each bile acid species can be calculated by performing linear regression. The ratio between the integrated bile acid peak and the integrated internal standard is calculated, after which the absolute amount can be calculated from the standard curve.

29. Calculate daily dietary cholesterol input according to the following formula:

$$^{diet}I = \frac{w_t - w_0}{\Delta t} \cdot {}^{diet}[cholesterol]$$

^{diet}I: dietary cholesterol intake ($\mu mol \cdot day^{-1}$)
w_t: weight (mass) of food at time point $t(g)$
w_0: weight (mass) of food at time point 0 (g)
t: time (days)
$^{diet}[cholesterol]$: cholesterol content in the food ($\mu mol/g$)

Table 1 Gas Chromatography Parameters for Measuring Cholesterol, Neutral Sterols, and Bile Acids

Injection parameters	
Injection technique	Splitless/split
Injection volume (µl)	1-3
Injection temperature (°C)	280
Rinsing liquid type	Heptane
Column parameters	
Column	Chromapack CPSil19 (or comparable): length 25 m, internal diameter, 0.25 mm; film thickness, 0.2 µm
Initial temperature (°C)	150 (splitless), 240 (split)
Initial time (min)	3 (splitless), 4 (split)
Initial program rate (°C/min)	20 (splitless), 10 (split)
Final temperature (°C)	280 (splitless/split)
Split final time (min)	16 (split)
Split final program rate (°C/min)	10 (split)
Split final temperature(°C)	290 (split)
Final time (min)	21 (splitless), 5 (split)
Run time (min)	30.5 (splitless), 29 (split)
Flow parameters	
Carrier gas type	Helium
Front pressure (kPa)	16 (constant pressure)
Average velocity (cm/sec)	31
Initial flow (ml/min)	0.9

30. Calculate daily fecal neutral sterol efflux according to the following formula:

$$^{NS}E = \frac{w_{feces}}{\Delta t} \cdot {}^{feces}[neutral\ sterols]$$

^{NS}E: fecal neutral sterol excretion ($\mu mol \cdot day^{-1}$)
w_{feces}: weight (mass) of feces (g)
$^{feces}[neutral\ sterols]$: neutral sterol content in feces ($\mu mol/g$)

31. Calculate daily fecal bile acid efflux according to the following formula:

$$^{BA}E = \frac{w_{feces}}{\Delta t} \cdot {}^{feces}[bile\ acids]$$

^{BA}E: fecal bile acid efflux ($\mu mol\ day^{-1}$)
$^{feces}[bile\ acids]$: bile acid content in feces ($\mu mol/g$)

FRACTIONAL CHOLESTEROL ABSORPTION USING DUAL TRACERS

BASIC PROTOCOL 2

Cholesterol is absorbed from the small intestinal lumen via NPC1L1 protein. In the past, cholesterol absorption was assessed by infusing radiolabeled cholesterol into the duodenum, after which lymph was sampled. This method became known as the 'direct method', due to the direct measurement of cholesterol passing through the duodenum. The necessity of sampling lymph makes the direct method impractical in humans and in

certain animal models. Indirect methods assess cholesterol absorption without the need for lymph, instead using fecal and/or blood samples, which can be obtained less invasively and over a longer time. We have previously described methods of estimating cholesterol absorption rates using both direct and indirect methods (Brufau and Groen, 2011). An example of an accessible indirect method is the sitostanol method, whereby labeled cholesterol and labeled sitostanol (which is assumed not to be absorbed) is ingested and measured in the feces (Brufau and Groen, 2011). The presence of sitostanol inside the intestinal lumen could interfere with the active process of cholesterol absorption and thereby with the measurement of fractional absorption (Heinemann et al., 1991). No substance or tracer used for the measurement of a biological process should ever influence the process itself. In this article, we describe an indirect dual-tracer tracer method, based on the study of Bosner et al. (1993) and Dijk (2010). In the past, we used radioactively labeled cholesterol tracers (van der Veen et al., 2005, 2009), but the advent of MIDA allowed us to fully utilize stable isotope tracers. Herein, labeled cholesterol is administered orally and intravenously, followed by blood sampling over several days. The ratio of oral to intravenously administered tracers is used to calculate the fractional cholesterol absorption rate. The benefit of performing a dual-tracer study compared to other methods such as the sitostanol method is the robustness and the possibility of estimating kinetics properties, cholesterol pool size, and fractional turnover rate.

Cholesterol absorption is calculated based on curve fitting of the plasma levels of orally and intravenously (i.v.) administered cholesterol tracers. It is assumed that the kinetics of the cholesterol tracers are identical to the unlabeled cholesterol. Theoretically, any two cholesterol tracers are suitable for performing a dual-tracer study. However, the two cholesterol tracers must (1) be distinguishable from one another with available equipment and (2) not interfere with the optional measurement of cholesterol synthesis. We have successfully used D_5 (2,2,4,4,6-deuterium-cholesterol)– and D_7 (25,26,26,26,27,27,27)–labeled cholesterol for assessing cholesterol absorption in combination with cholesterol synthesis (de Vogel-van den Bosch et al., 2008; Brufau and Groen, 2011; Brufau et al., 2011; Freark de Boer et al., 2012; Grefhorst et al., 2012; van der Wulp et al., 2012a,b; Dikkers et al., 2014). In experiments where cholesterol synthesis was not measured, we have successfully used ^{13}C2 cholesterol as an alternative to D_7-cholesterol. For the purpose of the experiment, it makes no difference whether D_5 is injected i.v. and D_7 is ingested orally, or vice versa. However, the use of D_7 cholesterol, or any labeled cholesterol that has its label at the C25-C27 position, is not advised if the method described herein is to be used and built upon for measuring newly synthesized bile acids. The reason for this is that C25-C27 (of cholesterol), and any tracer attached to these carbons, are cleaved off during the conversion of cholesterol to bile acids. After the administration of the tracers, blood is sampled at precisely indicated time points by serial blood-spot collection. The advantages of the collection of blood spots compared to non-bloodspot blood sampling techniques are the limited volume of blood that needs to be withdrawn from the rodent and the minimal amount of stress involved in the sampling procedure. Blood spot collection does not require anaesthesia nor restraint, and is therefore less stressful to the animal compared to retro-orbital or I.V. blood collection. Thereby, plasma parameters are only minutely affected, and ethical concerns for animal welfare are kept in order. The blood spots, approximately 6 mm in size, are collected from the tail on filter paper and dried at room temperature. When dried, they are called dried blood spots (DBS). The utilization of DBS allows for repeated sampling over time, and thus is suitable for obtaining kinetic data from individual animals (van der Veen et al., 2009; Brufau et al., 2011; Freark de Boer et al., 2012; Grefhorst et al., 2012; Dikkers et al., 2014). Each blood spot is approximately 10 µl in volume (van Dijk et al., 2003), resulting in a total blood volume of approximately 150 µl being drawn over the duration of the experiment. It has been empirically found that the appearance of cholesterol

in the sampled pool, i.e., blood, can be reliably described by an exponential growth curve. Cholesterol efflux from the sampled pool can be fit to two inverse exponential curves. The curves describe distinct biological processes that operate at different plasma disappearance (and perhaps elimination) rates.

Materials

D_5-cholesterol (2,2,4,4,6-deuterium-cholesterol; Medical Isotopes, Inc., cat. no. D201)
Absolute ethanol
D_7-cholesterol (25,26,26,26,27,27,27-deuterium-cholesterol; Medical Isotopes Inc, cat. No. D897)
20% Intralipid (Fresenius Kabi, or equivalent)
Medium-chain triglyceride (MCT) oil (SHS International, Nutricia, or equivalent)
Appropriate animal model (mouse or rat)Acetone
N_2 source
N,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco, cat. no. 33027)
Trimethylchlorosilane (TMCS; Thermo, cat. no. TS-88530)
Heptane

40º and 50ºC heat blocks or water baths
Rollerbank (Stuart Roller mixer, or equivalent)
Sample carrier filter paper (TFN grade, 182 g/m^2; Sartorius Stedim or equivalent)
Single-hole puncher (diameter ~6 mm)
2-ml microcentrifuge tubes
10-ml glass tube
GC/MS vials containing glass inserts (vials: Aluglas, cat. no. 1013679; inserts: Aluglas, cat. no. 1013586; caps: VWR, cat. no. 548-0085)
GC/MS system
SAAM II software (version 1.2.1 SAAM Institute, University of Washington, or version 2.3 The Epsilon Group)

Additional reagents and equipment for injection of mice (Donovan and Brown, 2006)

Prepare i.v. injection solution

1. Weigh 0.3 mg D_5-cholesterol per mouse. If necessary, correct for the estimated purity of the compound as indicated by the manufacturer.

   For later calculations, the exact fractional content of the isotopologs is assessed with GC/MS. Empirically we determined that the amount of tracer does not need to be adjusted for the body weight if the body weight falls within the standard range for non-obese mice. However, in case of very obese mice, it is advised to adjust the dosage of both the i.v. and oral administrations to the body weight to avoid signal-to-noise issues that arise when the tracers are further diluted inside the system.

2. Dissolve the D_5-cholesterol in absolute ethanol to a concentration of 25 mg/ml.

3. Heat the ethanol solution of D_5 and the 20% Intralipid to 40°C to accelerate solubilization.

4. Add the dissolved cholesterol to the Intralipid in a dropwise fashion for a final volume of 150 µl per animal. Vortex vigorously and place on a rollerbank until use. Keep a portion of the solution for GC/MS analysis.

Prepare oral gavage solution

5. Weigh 0.6 mg D_7-cholesterol per animal. If necessary correct for the estimated purity of the compound as indicated by the manufacturer.

Empirically, we have found that the quantity of this tracer has to be double the amount of the i.v. tracer.

6. Dissolve the D_7-cholesterol in medium-chain triglyceride oil, yielding 3 mg/ml in a final volume of 200 µl per animal. Place on a roller bank until use. Keep a remainder of the solution for GC/MS analysis.

7. At time point zero ($t = 0$ hr), take a blood spot from the tail of the test animal. Blood flow is started by making a small snip at the tail tip with iris scissors. Monitor the animal for excess bleeding. Collect the blood on sample carrier filter paper. Aim for a blood spot approximately 6 mm in diameter.

   The blood must be visible on both sides of the filter.

Administer cholesterol solutions and obtain blood samples

8. Immediately afterwards, perform oral gavage and i.v. injection (retro-orbital; Donovan and Brown, 2006) of D_7-cholesterol and D_5-cholesterol solutions, respectively.

9. Take blood spots at time point $t = 3$ hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr, 96 hr, 120 hr, 144 hr, 168 hr, 192 hr, 204 hr, 216 hr, 228 hr, and 240 hr. Blood flow is restarted by gently wiping the tail-tip wound. If blood flow cannot readily be restarted, the wound can be reopened with iris scissors. Monitor the animal for excess bleeding.

   Time points 0 hr to 168 hr are typically used for measuring cholesterol absorption. Time points 168 hr to 240 hr are typically used for measuring cholesterol synthesis (see Basic Protocol 3).

10. Allow blood spots to air dry for 2 hr at room temperature.

    DBS can be stored at room temperature for several months without degradation of the signal.

11. Remove the blood spot from the filter paper with a single-hole puncher (diameter ~6 mm), whereby the whole blood spot is removed from the filter paper. Transfer the punched circle to a 2-ml microcentrifuge tube.

    Due to the relative nature of dual-tracer measurements, it is not necessary to extract a consistent amount of blood from the filter paper, nor from the animal per se. D_5 and D_7 measurements (and any alternative tracers used) are expressed as a fraction of total cholesterol, which does not change with absolute blood volume. However, the amount of blood extracted from the filter paper should be sufficient for accurate GC/MS analysis. The signal-to-noise ratio will be lower when less blood is used.

The following procedure is performed for isolating and measuring cholesterol tracers using GC/MS, based on the work of Neese et al. (1993)

12. Add 25 µl Milli-Q water directly onto the blood spot (which is positioned on filter paper inside a 2-ml centrifuge tube). Close the tube air-tight.

13. Incubate at room temperature for at least 10 min.

    Older blood spots usually require longer incubation time to become completely wet.

14. Add 1.0 ml absolute ethanol:acetone (1:1 ratio). Close the tube air-tight, vortex vigorously, and incubate overnight at room temperature.

    The filter paper remains inside the tube throughout the procedure.

15. Centrifuge 10 min at 20,000 × *g*, room temperature.

16. Transfer 800 µl of the solution into a 10-ml glass tube and evaporate the fluid at 50°C under a stream of N_2 gas. When completely dry, let the tubes cool down to room temperature.

The remaining 200 µl (and the paper filter) serve as backup and can be stored at room temperature.

17. Add 100 µl BSTFA:TMCS (100:1) to the residue present in the 10 ml glass tube, incubate at room temperature overnight.

18. Evaporate at room temperature under N_2; keep the samples away from humidity.

19. Immediately add 100 µl heptane:BSTFA (100:1) to the sample residue and transfer the solution into a GC/MS vial containing a glass insert.

    For the evaluation of the GC/MS measurements, both the stability and the linearity of the GC/MS system have to be assessed. We advise measuring the fractional cholesterol distribution of samples per animal consecutively, followed by the control sample. Once every five animals, a dilution series is included. The repeated measurements of the control sample allow for the assessment of a drift in the GC/MS system. The relative standard deviation of the control samples should be less than 0.2% for m_0, and less than 0.3% for m_1. The sample series must be re-run upon exceeding this error. The control samples, derived from $t = 0$ hr before the administration of labeled cholesterol, represent natural enrichment. Thus, the control contains little m_3-m_7, leading to a higher relative standard deviation that should not be used for quality assessment. The repeated measurements of a dilution series allow for the assessment of the linearity of the GC/MS system. The relative standard deviation should be less than 0.4% for m_0, and less than 0.6% for m_1. All sample measurements must be within the linear dynamic range of the dilution series. Sample series must be rerun upon nonlinearity or if samples are not measured inside the dynamic range of the dilution series. It is advisable to measure the dilution series from low to high concentration, followed by a return to low to prevent any high-concentrate carry-over into subsequent samples. The injection volume of the samples can be changed to ensure they are measured within the linear range.

20. Prepare a pooled control sample and a pooled dilution curve:

    Control: Pool 10 µl of the $t = 0$ hr samples from all animals. The control sample represents the average natural enrichment of cholesterol within the cohort and is used for evaluating the stability of the GC/MS measurements. Additionally, the control sample can be used as a replacement for any missing $t = 0$ hr samples.

    Dilution series: Pool 10-µl samples from the time points with the highest enrichment values (3 hr to 48 hr) of all mice to a total of 1600 µl. In small cohorts, additional time points (72 hr to 168 hr) can be included. Evaporate the pool at 50°C under N_2, then redissolve in 200 µl heptane:BSTFA (100:1). Serially dilute by transferring 100 µl and diluting with 100 µl heptane:BSTFA (100:1) six consecutive times. The dilution series gives a total dynamic range of 64× (8×-4×-2×-1×-0.5×-0.25×-0.125×). To prevent contamination of the sensor and any carry-over to subsequent samples, the standard curve is measured low-high-low. In this way, the linearity of the GC/MS system is assessed.

21. Perform GC/MS analysis.

    Parameters for GC/MS analysis are described in Table 2 (van der Veen et al., 2009).

Mass spectrometry data normalization

Cholesterol ($C_{27}H_{46}O$) itself is composed of carbon, hydrogen, and oxygen, all of which have their own naturally occurring stable isotopes. From this, by chance alone, less than 75% of all cholesterol molecules exclusively contain ^{12}C, ^{1}H, and ^{16}O. The remainder of all cholesterol is distributed across various combinations of ^{13}C, ^{2}H, ^{17}O, and ^{18}O. The natural abundances are approximately ^{13}C: 1.11%; ^{2}H: 0.0115%; ^{17}O: 0.038%; and ^{18}O: 0.205%. Empirically, we have determined that correction for ^{13}C is sufficient.

Table 2 Gas Chromatography and Mass Spectrometry Parameters for Measuring Cholesterol-TMS Isotopologs

Injection parameters	
Injection mode	Pulsed splitless, front SS inlet He
Injection volume (µl)	4
Injection temperature (°C)	280
Septum purge flow (ml/min)	3
Column parameters	
Column	Rxi-5Sil-MS (or comparable); length, 30 m; internal diameter,0.25 mm,; film thickness, 0.25 µm
Initial temperature (°C)	150
Total flow (ml/min)	54.445
Holdup time (min)	1.093
Rise rate (°C/min)	40
Final temperature (°C)	280
Hold time (min)	8
Flow parameters	
Carrier gas type	Helium
Pressure (PSI)	19
Average velocity (cm/sec)	45.745
Mass spectrometry parameters	
Acquisition mode	SIM
Number of ions	8
Ions	458, 459, 460, 461, 462, 463, 464, 465
Dwell time	40
Solvent delay (min)	8
EM setting gain	4
MS source (°C)	230 (maximum: 250)
MS quad (°C)	150 (maximum 200)

Samples are analyzed by GC/MS. The monitored ions are 458 to 465 m/z (mass-to-charge ratio). These ions represent the cholesterol-TMS isotopomer distribution (m_0-m_7), which has to be corrected for natural abundance of ^{13}C by multiple linear regression as described by Lee and colleagues (Lee et al., 1991; Neese et al., 1993; van der Veen et al., 2009). For purposes of clarity, the calculations are described in the Supporting Material.

Calculation of the mathematical model used to describe cholesterol kinetics

Measurements performed by GC/MS give an estimation of the fractional contribution of the cholesterol tracers (such as D_5-cholesterol and D_7-cholesterol) in blood relative to the total amount of cholesterol (van der Veen et al., 2009). D_5-cholesterol is administered i.v. (via retro-orbital injection or other I.V. routes) and thus enters the sampled pool more rapidly compared to D_7-cholesterol, which is administered by oral gavage. There is a delay in oral-tracer blood entrance caused by various aspects of gastrointestinal motility, digestion, and absorption. The fractional cholesterol absorption can either be calculated at a single time point [such as $t = 72$ hr (Brufau and Groen, 2011)], but more robustly using the area under the curve (AUC). The AUC is calculated by estimating the fractional contribution of the tracer at time point zero (f), and the fractional elimination rate (k).

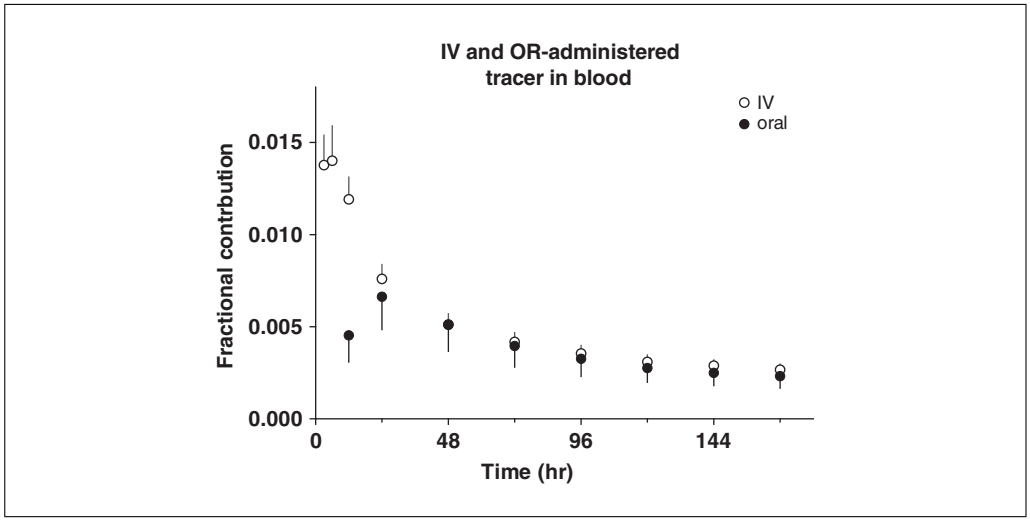

Figure 3 The fractions of i.v.- and oral-administered cholesterol tracer rapidly increase after administration, followed by a rapid and a mild decrease. The tracers are expressed as a fraction of the total free cholesterol in the sampled pool (blood). The appearance of the oral tracer in the sampled pool is delayed due to the effect of the gastrointestinal tract. The area under the curve is used to calculate the fractional cholesterol absorption. Kinetic parameters obtained from the curves are used to calculate the pool size of the sampled pool.

According to research performed by Hellerstein (1995), the data represents free plasma cholesterol which is affected by: plasma efflux to the different organs, conversion to non-free cholesterol (such as cholesteryl ester), and consumption by bile acid, vitamin, and steroid hormone production. The plasma efflux contains both a bidirectional and a unidirectional element. Bidirectional processes that exceed the experimental period are calculated as unidirectional processes. Empirically, it was seen that during an experiment, the fractional contribution of both tracers can be described by a three-phase exponential curve (Fig. 3) that comprises a rapid exponential rise, followed by a rapid, and a mild exponential, decline. The different parts of the curve are caused by a wide array of possible biological processes. The equations used for calculations were adapted from Rowland and Tozer (1995) and are described in detail below.

The data points, which represent the fractional contribution of the cholesterol tracer, can be fit in a three-phase curve described by:

$$f^{tr}(t) = f_1^{tr} e^{-k_1^{tr} \cdot t} + f_2^{tr} e^{-k_2^{tr} \cdot t} - f_3^{tr} e^{-k_3^{tr} \cdot t}$$

$f^{tr}(t)$: Fractional contribution of cholesterol tracer at time point t (no unit)
f_{1-3}^{tr}: Fractional contribution of cholesterol tracer at time point zero (no unit)
k_{1-3}^{tr}: Fractional elimination rate of the tracer (hr^{-1})

The i.v. tracer is introduced into the sampled pool and diluted instantaneously; therefore, the i.v. curve intersects the origin. The model describes the whole cholesterol course in a balanced system; thus f_3 (the input) must equal the sum of f_1 and f_2 (the output).

The kinetic parameters (f_x and k_x values) are estimated using curve-fitting software, such as SAAM II or R. Typical curve-fitting software will require initial values for the input parameters. Initial values are used by the non-linear least squares algorithm to solve the equation within a reasonable timeframe. During curve-fitting, it is possible that k_1 and k_2 are interchanged, resulting in the interchange of f_1 and f_2. Initial values are adjusted to assure $k_1 > k_2$.

The oral tracer enters the plasma with a delay in t. The delay (d) can be empirically estimated and inserted into the equation as a constant, or added to the model as $t - d$, to approximate the delay in uptake.

$$f^{tr}(t) = f_1^{tr} e^{-k_1^{tr} \cdot (t-d)} + f_2^{tr} e^{-k_2^{tr} \cdot (t-d)} - f_3^{tr} e^{-k_3^{tr} \cdot (t-d)}$$

It is advisable to explore the dataset as a whole and per group and to validate the kinetic parameters to the whole dataset (van Dijk et al., 2013). This is done by averaging the whole cohort and estimating the kinetic parameters. The kinetic parameters that are obtained from the cohort average are used as Bayesian values for fitting the averaged data of each experimental group. The average of all groups combined must be equal to the cohort average. The estimated kinetic parameters of each group are accepted when no significant difference remains between the parameters of the cohort and the averaged parameters of all groups. If a difference remains between the kinetic parameters of the whole dataset and the averaged parameters of all groups, the procedure is repeated with adjusted initial values. Next, the kinetic parameters are estimated per animal, whereby initial and Bayesian parameters obtained from groups are used. It is advised to repeat the procedure with adjusted initial parameters if the averaged kinetic parameters of all animals are not similar to the parameters of the whole dataset.

Calculation of the area under the curve

Area under the curve (AUC) is calculated according to the equation shown below. The curve continues its slope until the fractional contribution equals zero according to the model. Based on empirical data,~33% of the AUC is extrapolated if the latest time point is $t = 168$ hr. The curve does not approach zero until at least $t = 400$ hr. AUC for both the i.v. and the oral gavage curve is calculated with the following formula:

$$AUC^{tr} = \frac{f_1^{tr}}{k_1^{tr}} + \frac{f_2^{tr}}{k_2^{tr}} - \frac{f_3^{tr}}{k_3^{tr}}$$

AUC^{tr}: Area under the curve (hr)

Calculation of fractional cholesterol absorption

Fractional cholesterol absorption (F_{abs}) describes the fraction of orally administered (labeled) cholesterol that is absorbed from the gastrointestinal tract. It is calculated by dividing the AUC of the oral and i.v. curve, and correcting for differences in i.v. and oral dosage. For this, it is important that the fractional contributions of tracers in both dosages be measured by GC/MS. In further calculations, it is assumed that F_{abs} of dietary cholesterol is identical to that of biliary cholesterol and non-biliary cholesterol. However, it is possible that biliary cholesterol and TICE have a different fractional absorption compared to the dietary cholesterol. The fractional absorption of cholesterol is used to calculate the net cholesterol absorption rate (${}^{\text{int}\ es}_{plasma}E$) of the organism, based on the neutral sterol excretion (${}^{feces}E$).

$$F_{abs} = F^{IV} = \frac{AUC^{oral}}{AUC^{IV}} \cdot \frac{D^{IV}}{D^{oral}} \qquad {}^{intes}_{plasma}E = {}^{feces}E \cdot \frac{F_{abs}}{(1 - F_{abs})}$$

F_{abs}: Fractional absorption (no unit)
$D^{\text{IV·oral}}$: Tracer dosage (μ mol)
${}^{intestines}_{plasma}E$: Cholesterol absorption rate ($\mu\text{mol} \cdot \text{day}^{-1}$)
${}^{feces}E$: Fecal neutral sterol excretion rate ($\mu\text{mol} \cdot \text{day}^{-1}$)

Calculation of the total gastrointestinal efflux of cholesterol

Dietary cholesterol is subject to the fractional cholesterol absorption rate (F_{abs}), resulting in a fraction ($1 - F_{abs}$) being secreted into the feces. Total gastrointestinal efflux of cholesterol from the system can be calculated by correcting fecal neutral sterol efflux by the fractional absorption rate. Herein the assumption is made that, in contrast to what has been discussed above, all fecal neutral sterol components are subject to the same fractional absorption.

$$^{diet}E = {}^{diet}I \cdot (1 - F_{abs}) \qquad {}^{total}E = \frac{{}^{feces}E}{F_{abs}}$$

^{diet}E: Fecal cholesterol originating from the diet ($\mu mol \cdot day^{-1}$)
$^{total}E$: Total gastrointestinal efflux of cholesterol ($\mu mol \cdot day^{-1}$)

Calculation of plasma cholesterol turnover rate

Plasma cholesterol turnover rate (*Ra*) describes the rate at which cholesterol enters and is removed from the sampled plasma pool at steady state. It is calculated as the ratio of the administered i.v. dose corrected for its bioavailability (*F*) and the AUC of the tracer. The bioavailability of an i.v.-injected substance (F^{IV}) is by definition set to 1 because it is directly administered into the sampled pool. The cholesterol pool size describes the total amount of cholesterol in the sampled pool and is calculated as the ratio of the administered i.v. dose corrected for its bioavailability and the fractional contribution of the cholesterol tracer ($f_3{}^{IV}$) entering the sampled pool.

$$Ra = \frac{F^{IV} \cdot D^{IV} \cdot 24}{AUC^{IV}} \qquad Q = \frac{F^{IV} \cdot D^{IV}}{f_3^{IV}} \qquad K = \frac{Ra}{Q}$$

Ra: Plasma cholesterol turnover rate (μmol/day)
Q: Cholesterol pool size (μmol)
K: Fractional turnover rate of the sampled pool (day-1)

BASIC PROTOCOL 3

CHOLESTEROL SYNTHESIS MEASUREMENT USING LABELED ACETATE

Cholesterol is synthesized inside nearly all mammalian cell types by a series of enzymatic reactions that find their origin in acetate. Briefly, cholesterol is synthesized in a three-step process: (1) 3 mol of acetyl-CoA are used to produce 1 mol of isopentenyl pyrophosphate (IPP); (2) 6 mol of IPP are used to produce 1 mol of squalene; (3) squalene cyclizes and is converted into cholesterol. In several reaction steps, carbon atoms are lost as CO_2. In total, 1 mol of cholesterol requires 18 mol of acetyl-CoA. Within the reaction chain of cholesterol synthesis, several enriched pools will exist simultaneously. The net effect of all enriched pools is that statistically it appears that cholesterol is produced as a polymer of 12 (*n*) acetate molecules. The absolute cholesterol synthesis rate (E^{new}) can be determined from the fractional synthesis as calculated by mass isotopomer distribution analysis (MIDA) and the pool size (*Q*). The cholesterol synthesis protocol described in this paper is based on the work of Neese and colleagues (Neese et al., 1993) and Hellerstein (Hellerstein and Neese, 1992; Hellerstein, 1995; Hellerstein and Neese, 1999). In short, there is a unique relationship (formula shown below) between precursor pool (acetyl-CoA) enrichment (*p*) and the relative abundance of the isotopologs in the newly synthesized cholesterol fraction. This theoretical relationship can be calculated from a frequency distribution, and is described by polynomial expansion. The relationship between isotopologs consisting of one or more ^{13}C isotopes is conserved when they are diluted by pre-existing (non-labeled) cholesterol. The ratio of two or more isotopologs as measured by GC/MS is then used to estimate the precursor pool enrichment. From this,

the fractional contribution of the isotopologs in the newly synthesized pool is estimated. The fractional contribution of newly synthesized cholesterol in the sampled pool can be estimated as the ratio of the fractional contribution of one of the isotopologs in the sample and its estimated distribution in the newly synthesized fraction. A theoretical distribution table of newly synthesized cholesterol is calculated for M_0-M_4. The relative contribution of M_5 and beyond is negligible at typical enrichment values. Each M_x is divided by the sum of M_0-M_4 for correction. Empirically it was found that the ratios of the isotopologs M_1 and M_3 gave the most reliable results. For purposes of clarity, the calculations are described in the Supporting Material.

$$M_x^{new} = \frac{n!p^x(1-p)^{n-x}}{(n-x)!\,(x)!}$$

M_x^{new}: Theoretical abundance of isotopologs (no unit)
n: Number of subunits in the polymer (no unit)
p: Precursor pool enrichment (no unit)
x: Number of labeled subunits in the polymer (no unit)

Materials

Appropriate animal model (mouse, rat)
Sodium [1-^{13}C]acetate- (Sigma-Aldrich, cat. No. 668656, or equivalent)

Sterilization filter (0.2-µm, for sterilizing acetate solutions)
SAAM II software (version 1.2.1 SAAM Institute, University of Washington, or or version 2.3 The Epsilon Group)

Additional reagents and equipment for preparing blood spots (Basic Protocol 2)

1. Prepare drinking water with 2% (w/v) labeled acetate (75 ml per mouse).
2. Sterilize the water by filtering it through a 0.2-µm filter.
3. Exchange the drinking water of the animals with the labeled acetate/water on $t =$ 168 hr (right after drawing a blood spot from the tail).

   The water is not changed for the remainder of the experiment. Leftover water can be filtered with a 0.2 µm filter and stored at −20°C for later use.

4. Take blood spots (see Basic Protocol 2) at $t =$ 168 hr, 192 hr, 204 hr, 216 hr, 228 hr, and 240 hr.

   The time points and the methodology are compatible with the cholesterol absorption method described earlier in this paper.

5. Allow blood spots to air dry for 2 hr at room temperature.

   The dried blood spots can be stored at room temperature for several months without degradation of the signal.

6. Isolate and measure cholesterol isotopologs as described in Basic Protocol 2.
7. Calculate the fractional contribution of ^{13}C-labeled cholesterol (f_t^{new}) at each time point (see Supporting Material) and perform curve-fitting using software such as SAAM II or R.

   Each animal ingested a certain amount of ^{13}C-labeled acetate and has a certain body weight, both being factors that affect the amount of tracer inside the system and the ultimate concentration (enrichment) of the tracer relative to total cholesterol. Thereby, the exact precursor pool enrichment (p) will be different for each animal. It is necessary to calculate the precursor pool enrichment for each animal individually

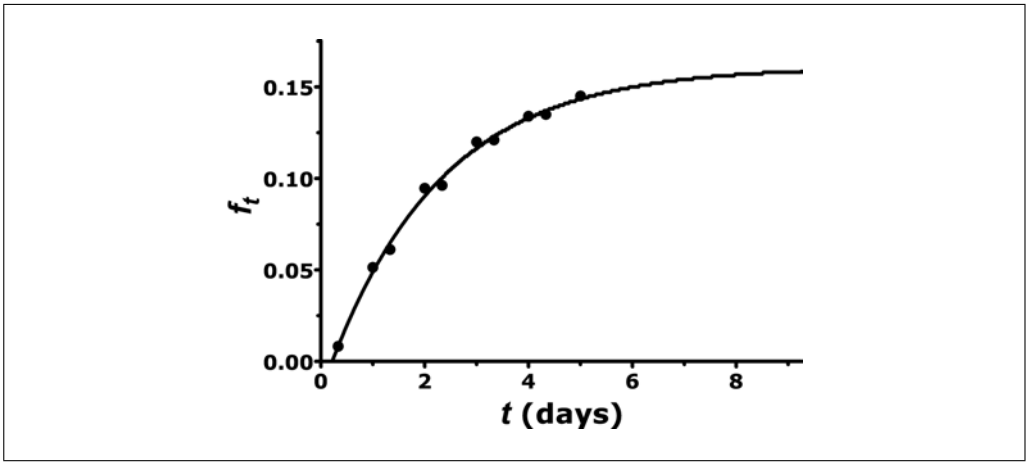

Figure 4 The fraction de novo synthesized cholesterol rapidly increases and eventually plateaus when an equilibrium in the product pool (^{13}C-cholesterol) is reached after approximately 4 days. The plateau represents the fractional synthesis rate at steady state and is used for calculating the absolute cholesterol synthesis rate.

based on Lee-corrected M_1 and M_3 values (Hellerstein and Neese, 1992; Hellerstein, 1995; Hellerstein and Neese, 1999). Precursor pool enrichment is calculated by performing polynomial regression on a selection of the polynomial-expanded data. The theoretical maximum fractional contribution ($M_1{}^{new}$ and $M_3{}^{new}$) is calculated based on the pool enrichment by performing polynomial regression on a selection of the polynomial-expanded data. The fractional contribution of ^{13}C-labeled cholesterol (f_t^{new}) is calculated with the formula show below (see Supporting Material for the complete calculation sheet).

$$f_t^{new} = \frac{M_1}{M_1^{new}} = \frac{M_3}{M_3^{new}}$$

f_t^{new}: Fraction de novo synthesized cholesterol at time point t (no unit)

It has been empirically determined that the isotopic steady state in the acetyl-coA precursor pool (that is, the even and stable distribution of labeled acetate across all relevant body pools of acetate) is reached within a time frame of hours. It takes several days (usually in the order of 4–5 days) to reach an isotopic steady state in the product pool, i.e., ^{13}C-labeled cholesterol. The fractional synthesis rate at steady state (F^{new}), as well as the fractional turnover rate (k), can be calculated by modeling the data. The model describes the rise in fractional-contribution of ^{13}C-labeled cholesterol as the steady state is forming (Fig. 4).

$$f_t^{new} = F^{new} \cdot \left(1 - e^{-kt}\right)$$

The absolute cholesterol synthesis rate is calculated from the fractional synthesis rate at steady state (F^{new}), the turnover rate (k), and the cholesterol pool size (Q). The cholesterol pool size is obtained from the cholesterol absorption kinetics data, but can alternatively be obtained from literature (Dietschy and Turley, 2002).

$$E^{new} = F^{new} \cdot k \cdot Q$$

E^{new}: Absolute cholesterol synthesis rate (μmol · day-1)
F^{new}: Fractional synthesis rate at steady state (no unit)
Q: Cholesterol pool size (μmol)

BILIARY AND NON-BILIARY CHOLESTEROL EXCRETION

The major cholesterol efflux routes are via the bile (^{bile}E; Hill and McQueen, 1997) and via TICE (Temel and Brown, 2012). Blood cholesterol–synthesized and hepatic-synthesized cholesterol are exported into the bile. Bile flow is measured by performing bile-duct cannulation (Kuipers et al., 1996). To measure biliary cholesterol excretion, bile flow and biliary cholesterol concentration have to be measured. Bile is collected for a set period of time (exactly 30 min) and the cholesterol concentration is measured. The interruption of the enterohepatic cycling of bile salts will lead to depletion of the bile salt pool and in turn induce a decrease in bile flow. For this reason, it is critical to always use the same sampling duration within and between experiments.

Aside from the biliary route, cholesterol can also be removed from the system via a non-biliary route. In ABCG5/G8$^{-/-}$, Mdr2$^{-/-}$, and NPC1L1$^{-liverTg}$ animals, wherein biliary cholesterol secretion is severely diminished, the fecal neutral sterol output is only modestly decreased (Temel and Brown, 2012). These findings imply that a non-biliary mechanism exists and is able to compensate for biliary dysfunction. A process that has become known as transintestinal cholesterol efflux (TICE) provides an alternative non-biliary route, which can be potently stimulated by biliary insufficiencies and by drug treatment.

Materials

Appropriate animal model (mouse)
Chloroform
Methanol
Triton X-100
Nitrogen source
Absolute ethanol
0.2 M potassium phosphate buffer, pH 7.4
Sodium cholate
4-hydroxyphenylacetic acid (Sigma, cat. no. H50004)
Cholesterol oxidase enzyme (Sigma, cat. no. 26746 or C8868, or equivalent)
Cholesterol peroxidase enzyme (Roche, cat. no. 10108090001 or Sigma, cat. no. P8250 or equivalent)
Cholesterol esterase enzyme (Sigma, cat. no. C9281)
5β-Cholanic acid-7α, 12α-diol (Steraloids, cat. no. C1170)
Bile
0.1 M sodium acetate buffer, pH 5.6
Choloyl glycine hydrolase (Sigma, cat. no. C4018)

0.5-ml tubes, labeled
Scale with 0.1 mg accuracy (to weigh tubes)
Gallbladder cannula
37ºC incubator, or heat mat
Scale with 0.1 g accuracy (to weigh mice)
Incubator or heat pad to maintain body temperature
Container to accommodate the animal during weighing
ELISA Plate Reader capable of 340 nm excitation and 420 nm emission
Black non-transparent 96-wells ELISA plates

Additional reagents and equipment for determining bile acid profile by GC (Basic Protocol 1)

1. Weigh labeled tubes (0.5 ml, preferably to 0.1-mg accuracy) that will be used for bile collection and record the exact weights.

Perform bile cannulation

Within an experiment, bile cannulation should be performed at the same period of the day for all animals due to circadian differences in bile production. The bile cannulation procedure is performed as described by Kuipers et al. (1996).

2. In brief, weigh the animal, anesthetize the animal, place a cannula inside the gallbladder, and ligate the common bile duct (which transports bile from the liver and the gallbladder to the duodenum). Place the animal inside a 37°C incubator or on a heat mat to maintain constant body temperature.

   Body temperature is affected by anesthesia and affects bile flow.

3. When bile flow is successfully attained, discard the first 5 min of flow.

   The initial flow originates from the gallbladder and is not representative of the true bile.

4. Collect bile into the weighed tubes for exactly 30 min.

   The sampling time must be kept identical between animals and between experiments.

5. Weigh the tubes to calculate the weight of the bile collected. Determine the density by pipetting an aliquot and weighing it.

6. Measure the cholesterol concentration by fluorimetry as follows. Dilute 15 µl of bile in 1.2 ml Milli-Q water in a 10-ml glass tube and add 4.5 ml chloroform:methanol (1:2 v/v). Vortex vigorously for 30 sec.

7. Add 1.5 ml of chloroform and vortex, followed by 1.6 ml of Milli-Q water. Vortex again.

8. Centrifuge 10 min at 1500 × *g*, room temperature.

9. Transfer the bottom chloroform layer into a clean 10-ml glass tube and evaporate the sample under N_2 at 50°C.

10. Redissolve the sample into 450 µl of chloroform and immediately pipet 300µl into a clean 10 ml glass tube for measuring the cholesterol concentration. The remaining 150 µl is kept as a backup.

11. Evaporate the 300 µl chloroform (containing the cholesterol sample) and redissolve in 100 µl absolute ethanol.

12. Prepare a standard curve of 0 to 40 µg/ml cholesterol in 100 µl absolute ethanol.

13. Without vortexing, prepare a master mix containing:

    3 ml Milli-Q water
    1 ml Triton X-100 (0.5%)
    14 ml 0.2 M phosphate buffer (K_2HPO_4, pH = 7.4)
    1 ml 20 mM sodium cholate
    12 mg 4-hydroxyphenylacetic acid
    2 U Cholesterol-oxidase enzyme
    20 U Peroxidase enzyme
    0.2 U Cholesterol-esterase enzyme (optional, to measure the amount of cholesterol-ester)

14. Add 600 µl of master mix to each sample and standard, gently mix, and incubate for 20 min in the dark at room temperature.

15. Transfer 250 µl to a black 96-well ELISA plate

16. Measure the fluorescent intensity (excitation: 340 nm, emission: 420 nm)

17. Prepare a linear standard curve and calculate the cholesterol concentration in the bile.

18. Measure the bile acid profile as follows. Add 13 nmol of 5β-Cholanic acid 7α,12α diol (internal standard for bile acids) to a 10-ml glass tube and evaporate the solvant under N_2 at 50°C.

19. Pipet 5 µl of bile into the tube and add 1.0 ml 0.1 M sodium acetate buffer (pH = 5.6).

20. Add 12 U choloyl glycine hydrolase enzyme and mix carefully without vortexing.

21. Incubate at 37°C for 15 hr and let the tube cool down to room temperature. Continue from step 18 in Basic Protocol 1.

22. Calculate the biliary cholesterol efflux by multiplying the flow by the cholesterol concentration, thereby giving an estimation of the amount of cholesterol exiting the system via the bile per day.

$$^{bile}E = bile\ flow \cdot ^{bile}[cholesterol]$$

^{bile}E: biliary cholesterol excretion (µmol · day-1)
bile flow: volume of collected bile (µl/day)
$^{bile}[cholesterol]$: cholesterol concentration in bile (µmol/µl)

23. Calculate the amount of bile-derived cholesterol that will end up in the feces by multiplying the flow and concentration by the fractional cholesterol absorption. Herein the assumption is made that bile-derived cholesterol absorption is identical to diet-derived cholesterol absorption, as measured with the dual tracer method.

$$^{bile}_{feces}E = bile\ flow \cdot ^{bile}[cholesterol] \cdot (1 - F_{abs})$$

24. Calculate the non-biliary cholesterol efflux, i.e., TICE, by determining the difference between the total amount of cholesterol that leaves the intestinal tract and the known amount of cholesterol that enters the tract (dietary and biliary). As such, TICE can be regarded as a net flux along the whole length of the intestine, i.e. the difference between influx into the intestine and efflux via the feces.

$$^{plasma}_{intes}E = ^{TICE}E = \left(^{total}E + ^{intes}_{plasma}E\right) - \left(^{diet}E + ^{bile}E\right)$$

^{TICE}E: non-biliary neutral sterol excretion (µmol day^{-1})

25. Estimate the whole-body cholesterol balance by calculating the gastrointestinal sterol balance Using the information obtained in this protocol, it is possible to estimate the whole-body cholesterol balance by calculating the gastrointestinal sterol balance. Thereby, the assumption is made that the amount of cholesterol lost in the urine, sebum, hair, tears, skin, and miscellaneous disposal sites remains constant during the duration of the experiment. The gastrointestinal sterol balance is calculated, whereby a positive balance indicates a perceived accumulation of cholesterol inside the system and a negative balance indicates a perceived net loss of cholesterol from the system.

$$^{balance}E = \left(^{diet}I + E^{new}\right) - \left(^{feces}E + ^{bile\,acids}E\right)$$

$^{balance}E$: Sterol balance (µmol day^{-1})

COMMENTARY

Background Information

This article is a direct continuation of our previous work on measuring cholesterol metabolism (Brufau and Groen, 2011). Compared to our previously published protocol, the methods described herein build upon many aspects previously not touched upon, such as cholesterol synthesis and pool size. Cholesterol absorption is often calculated from a single time point, measured by, for example, the fecal sitostanol or a dual-tracer method. In this protocol, cholesterol absorption is measured over a range of time, thereby increasing the robustness.

Critical Parameters

Animal housing

The number of animals per cage is a critical parameter in cholesterol flux studies. Individual housing of animals is the only viable method for the measurement of dietary intake and fecal neutral sterol output on a per-animal basis. The cage in which an animal is situated is regarded as a closed system, from which cholesterol is added via the diet and removed by changing the bedding material (thereby taking feces out of the cage). If more than one animal is placed inside a cage, it is no longer possible to measure cholesterol intake and excretion on a per-animal basis. In such an experimental setup, the data is pooled. Coprophagy in individually housed animals is not expected to affect cholesterol metabolism. However, the effect of coprophagy in groups of animals, whereby fecal neutral sterols and bile acids can be exchanged between animals, is not known. It is not known to what extent fecal material from cagemates is consumed. Group sizes should be adjusted for data pooling if individual housing is not possible or not desired.

Number of animals per group

Due to the nature of metabolic research, relatively high variability between animals is expected. Cholesterol absorption, excretion, synthesis and flux are systemic processes that involve complex inter-organ cross-talk. Careful planning and strictly controlled animal housing and experimental conditions are critical parameters for limiting variability as much as possible. GC/MS analyses require strict quality control measures. It is advised to use a minimum of eight animals per group to delineate subtle biological (treatment) effect from natural variability.

Chow diet

The composition of animal chow, especially the concentration of cholesterol and plant sterols, constitutes an important experimental parameter. Chow diet is only partially defined and is produced from agricultural products that are inherently variable between batches. Sterol content varies widely between batches and between manufacturers. It is strongly advised to perform experiments with a single batch of chow and to characterize the sterol levels of each batch separately before commencing experiments.

Animal discomfort

Measurement of food intake and fecal output requires individual housing of the animals. A sparse amount of bedding and nesting material is placed inside the cage, to allow for the collection of feces. Mice and rats (as well as virtually all rodents) are burrowing animals that exhibit nest-building and digging behavior. These behaviors interfere with the collection of feces and are prevented by limiting the amount of bedding and nesting material (sometimes called “cage enrichment”). The inability to express these behaviors causes a degree of stress in the animals that cannot be completely avoided. The animals are handled on a daily basis for more than 1 week for the collection of bedding, for measuring the body weight, and for sampling blood from the tail. Empirically, we have observed that in some experiments both male and female mice show a decreased weight gain over the course of the protocol. The effect on body weight is likely due to stress or discomfort and cannot be avoided completely. The protocol described herein was designed in such a way to cause the least amount of stress and discomfort. However, all procedures must first be approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations regarding the care and use of laboratory animals.

Blood cholesterol

Cholesterol obtained from the blood spots (the sampled pool) measured by GC/MS represents the free cholesterol. The described GC/MS protocol and measurement parameters were optimized for measuring cholesterol-TMS formed from free cholesterol.

Table 3 Troubleshooting Guide for Measurement of Cholesterol Fluxes

Problem	Cause	Solution
Feces quantity measurements have high variability	Not all feces are collected from the bedding	Use a minimum amount of bedding to facilitate rapid sorting of feces Improve visibility of the feces by utilizing a diet with a non-absorbed and non-degradable food dye
	Feces cling to bedding or cage enrichment	Do not use cotton or cotton-like material for bedding or cage enrichment. Large paper shreds and wood chips can be used as an alternative cage enrichment.
Food quantity measurements have high variability	Food is lost due to crumbling or wasting by the animals	Use a non-crumbling diet Manually sort food particles from the bedding In some instances unavoidable. Certain diets, such as high-fat diet, are inherently crumbling.
Bile parameters have high variability	Bile was collected during different parts of the day within one cohort	It is strongly advised to sample bile during the same period of the day for all animals due to differences in bile flow caused by circadian rhythm and the recent consumption of food Split cohorts up into several days to assure bile is sampled within a narrow time frame of the day
	Initial bile flow was not discarded	It is strongly advised to dispose of the first 5 min of bile after flow has been successfully attained. The first 5 min of bile originates from the gall bladder and is not representative of the bile itself. In animal species that do not have a gall bladder, such as the rat, this is not necessary.
	Collection time was not consistent between animals	Collect bile for exactly 30 min after the initial 5 min of flow have been discarded. If bile is collected in increments totaling 30 min, pool the samples on a per-animal basis to obtain a 30-min pool.
	Bile flow was not stable due to anesthesia or body temperature	It is advised to place the animal inside an incubator or alternatively on a heating pad to maintain a steady body temperature during bile sampling Maintain a steady flow of volatile anesthesia that is kept identical between animals
GC-MS data not within specifications	Technical fault in measurement system	Confirm GC-MS parameters, change parameters if faulty Check column integrity, replace column if necessary
	Data are not within linear range	Confirm that the system is linear within the dynamic range of the standard curve; adjust dynamic range if not Confirm that samples are within the standard curve; re-analyze samples by changing the injection volume if not
	System is not stable and drifts over time	Confirm that the system is stable to within specifications by comparing control samples over-time Reanalyze the series of samples if not
	Big differences in natural enrichment between groups	Animals were given a diet rich in ^{13}C (such as corn oil). Avoid differential quantities of ^{13}C between groups. Correct for natural enrichment on a per-animal basis.
Curve fitting not successful	Convergence issues due to an exceedance of the maximum number of iterations	Initial values for the parameters have to be chosen carefully. We advise to explore a dataset by foremost pooling all data and using obtained values either as a range or as a Bayesian mean. Adjust the initial values to facilitate curve fitting. Manually increase the maximum number of iterations and/or adapt other aspects of the non-linear least-squares method to facilitate fitting

continued

Table 3 Troubleshooting Guide for Measurement of Cholesterol Fluxes, *continued*

Problem	Cause	Solution
	Errors in data points severely disturb curve fitting	Adjust the weight of erroneous data by applying a fractional standard deviation to individual points Reanalyze data series, recalculate data, (not preferred) exclude data points
	1-^{13}C-acetate was given during cholesterol absorption assessment	Labeled acetate causes discrepancies in D5 and D7 measurements, decreasing the signal-to-noise ratio. Segregate cholesterol absorption and synthesis into separate non-overlapping parts of the experiment. Exclude data points that are affected by labeled acetate.
	Filter paper used for blood spots was contaminated	Avoid bare-skin contact as much as possible. Do not press the filter paper against the skin of the animal.
Unusual TICE values	Intervention has a major effect on intestinal shedding or intestinal cholesterol synthesis	Interpret results with caution if it is suspected that the intervention has a major effect on intestinal shedding or intestinal cholesterol synthesis

Intestinal shedding

The model used in this protocol combines trans-intestinal cholesterol efflux with intestinal shedding (E^{shed}) and de novo synthesis of cholesterol within the intestinal lining. Calculations obtained from any (pharmacological) treatment that is known to increase intestinal shedding or cholesterol synthesis should be interpreted with caution.

Curve fitting

The cholesterol absorption and synthesis calculations are both reliant on curve-fitting to estimate the kinetic parameters, which are used to estimate parameters of cholesterol metabolism. The quality of curve fitting is a critical parameter. Within this paper, SAAM II, MATLAB, and the R programming language have been suggested as tools for estimating the kinetic parameters. Other software solutions that allow for the estimation of multiple unknown parameters are likewise suitable.

Troubleshooting

Table 3 describes some problems commonly encountered with the protocols in this article along with explanations of the causes of these problems and recommendations for avoiding or overcoming them.

Anticipated Results

Plasma

Typical plasma total cholesterol levels (95% confidence interval) for C57Bl/6 mice are 1.24 to 2.57 mM, 0.15 to 0.71 mM free cholesterol, and 0.96 to 2.28 mM cholesteryl ester. Typical plasma total cholesterol levels for Wistar Unilever rats are 1.22 to 2.60 mM, and 0.16 to 1.44 mM free cholesterol.

Bile cannulation

Typical bile flow (95% confidence interval) for C57Bl/6 mice is 3.2 to 7.2 $\mu l \cdot min^{-1} \cdot 100\ g^{-1}$, free cholesterol is 1.5 to 6.1 $nmol \cdot min^{-1} \cdot 100\ g^{-1}$, phospholipids is 6.5 to 66.9 $nmol \cdot min^{-1} \cdot 100\ g^{-1}$, and bile salts is 59 to 775 $nmol \cdot min^{-1} \cdot 100\ g^{-1}$. Typical bile flow for Wistar Unilever rats is 5.3 to 6.5 $\mu l \cdot min^{-1} \cdot 100\ g^{-1}$, free cholesterol is 2.1 to 6.8 $nmol \cdot min^{-1} \cdot 100\ g^{-1}$, phospholipids is 19.4 to 38.9 $nmol \cdot min^{-1} \cdot 100\ g^{-1}$, and bile salts is 80 to 432 $nmol \cdot min^{-1} \cdot 100\ g^{-1}$.

Time Considerations

The protocol was designed in such a way that the animal work can be performed within 12 days. Due to the modular nature of the protocol, various adaptations can be made, such as the exclusion of cholesterol synthesis, thereby shortening the procedure to 9 days.

Supporting Materials

All supporting materials discussed in this article can only be accessed from the online version of this article.

Acknowledgments

The authors thank Theo Boer for extensive GC/MS work and optimization.

Conflict of Interest

The authors have declared no conflicts of interest for this article.

Literature Cited

Bosner, M.S., Ostlund, R.E., Osofisan, O., Grosklos, J., Fritschle, C., and Lange, L.G. 1993. Assessment of percent cholesterol absorption in humans with stable isotopes. *J. Lipid Res.* 34:1047-1053.

Brufau, G. and Groen, A.K. 2011. Characterization of whole body cholesterol fluxes in the mouse. *Curr. Protoc. Mouse Biol.* 1:413–427. doi: 10.1002/9780470942390.mo110118.

Brufau, G., Kuipers, F., Lin, Y., Trautwein, E.A., and Groen, A.K. 2011. A reappraisal of the mechanism by which plant sterols promote neutral sterol loss in mice. *PLoS ONE* 6:e21576. doi: 10.1371/journal.pone.0021576.

de Vogel-van den Bosch, H.M., de Wit, N.J.W., Hooiveld, G.J.E.J., Vermeulen, H., van der Veen, J.N., Houten, S.M., Kuipers, F., Müller, M., and van der Meer, R. 2008. A cholesterol-free, high-fat diet suppresses gene expression of cholesterol transporters in murine small intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294:G1171-G1180. doi: 10.1152/ajpgi.00360.2007.

Dietschy, J.M. and Turley, S.D. 2002. Control of cholesterol turnover in the mouse. *J. Biol. Chem.* 277:3801-3804. doi: 10.1074/jbc.R100057200.

Dikkers, A., Annema, W., de Boer, J.F., Iqbal, J., Hussain, M.M., and Tietge, U.J.F. 2014. Differential impact of hepatic deficiency and total body inhibition of microsomal triglyceride transfer protein on cholesterol metabolism and reverse cholesterol transport in mice. *J. Lipid Res.* 55:816-825. doi: 10.1194/jlr.M042986.

Donovan, J. and Brown, P. 2006. Parenteral injections. *Curr. Protoc. Immunol.* 73:1.6.1-1.6.10. doi: 10.1002/0471142735.im0106s73.

Fielding, C.J. and Fielding, P.E. 1995. Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* 36:211-228.

Freark de Boer, J., Annema, W., Schreurs, M., van der Veen, J.N., van der Giet, M., Nijstad, N., Kuipers, F., and Tietge, U.J.F. 2012. Type I diabetes mellitus decreases in vivo macrophage-to-feces reverse cholesterol transport despite increased biliary sterol secretion in mice. *J. Lipid Res.* 53:348-357. doi: 10.1194/jlr.M018671.

Grefhorst, A., Oosterveer, M.H., Brufau, G., Boesjes, M., Kuipers, F., and Groen, A.K. 2012. Pharmacological LXR activation reduces presence of SR-B1 in liver membranes contributing to LXR-mediated induction of HDL-cholesterol. *Atherosclerosis* 222:382-389. doi: 10.1016/j.atherosclerosis.2012.02.014.

Groen, A.K., Oude Elferink, R.P., Verkade, H.J., and Kuipers, F. 2004. The ins and outs of reverse cholesterol transport. *Ann. Med.* 36:135-145. doi: 10.1080/07853890310020635.

Heinecke, J.W. 2012. The not-so-simple HDL story: A new era for quantifying HDL and cardiovascular risk? *Nat. Med.* 18:1346-1347. doi: 10.1038/nm.2930.

Heinemann, T., Kullak-Ublick, G.-A., Pietruck, B., and Von Bergmann, K. 1991. Mechanisms of action of plant sterols on inhibition of cholesterol absorption. *Eur. J. Clin. Pharmacol.* 40:S59-S63. doi: 10.1007/BF03216292.

Hellerstein, M.K. 1995. Methods for measurement of fatty acid and cholesterol metabolism. *Curr. Opin. Lipidol.* 6:172-181. doi: 10.1097/00041433-199506000-00010.

Hellerstein, M. and Neese, R. 1992. Mass isotopomer distribution analysis: A technique for measuring biosynthesis and turnover of polymers. *Am. J. Physiol. Endocrinol. Metab.* 263:E988-1001.

Hellerstein, M.K. and Neese, R.A. 1999. Mass isotopomer distribution analysis at eight years: Theoretical, analytic, and experimental considerations. *Am. J. Physiol. Endocrinol. Metab.* 276:E1146-E1170.

Hill, S.A. and McQueen, M.J. 1997. Reverse cholesterol transport: A review of the process and its clinical implications. *Clin. Biochem.* 30:517-525. doi: 10.1016/S0009-9120(97)00098-2.

Ikonen, E. 2008. Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* 9:125-138. doi: 10.1038/nrm2336.

Jackson, K.A. and Topping, D.L. 1993. Prevention of coprophagy does not alter the hypocholesterolaemic effects of oat bran in the rat. *Br. J. Nutr.* 70:211-219. doi: 10.1079/BJN19930118.

Jakulj, L., Mohammed, H., van Dijk, T.H., Boer, T., Turner, S., Groen, A.K., Vissers, M.N., and Stroes, E.S.G. 2013. Plasma plant sterols serve as poor markers of cholesterol absorption in man. *J. Lipid Res.* 54:1144-1150. doi: 10.1194/jlr.P031021.

Kudchodkar, B.J., Sodhi, H.S., and Horlick, L. 1972. Lack of degradation of dietary and endogenous sterols in gastrointestinal tract of man. *Metabolism* 21:343-349. doi: 10.1016/0026-0495(72)90079-0.

Kuipers, F., van Ree, J.M., Hofker, M.H., Wolters, H., Veld, G.I., Havinga, R., Vonk, R.J., Princen, H.M., and Havekes, L.M. 1996. Altered lipid metabolism in apolipoprotein E–deficient mice does not affect cholesterol balance across the liver. *Hepatology* 24:241-247. doi: 10.1002/hep.510240138.

Lee, W.N.P., Byerley, L.O., Bergner, E.A., and Edmond, J. 1991. Mass isotopomer analysis: Theoretical and practical considerations. *Biol. Mass Spectrom.* 20:451-458. doi: 10.1002/bms.1200200804.

Neese, R.A., Faix, D., Kletke, C., Wu, K., Wang, A.C., Shackleton, C.H., and Hellerstein, M.K. 1993. Measurement of endogenous synthesis of plasma cholesterol in rats and humans using MIDA. *Am. J. Physiol. Endocrinol. Metab.* 264:E136-E147.

Nibbering, C.P., Groen, A.K., Ottenhoff, R., Brouwers, J.F.H.M., vanBerge-Henegouwen, G.P., and van Erpecum, K.J. 2001. Regulation of biliary cholesterol secretion is independent of hepatocyte canalicular membrane lipid composition: A study in the diosgenin-fed

rat model. *J. Hepatol.* 35:164-169. doi: 10.1016/S0168-8278(01)00125-8.

Rowland, M. and Tozer, T.N. 1995. Clinical Pharmacokinetics: Concepts and Applications. Lippincott Williams & Wilkins, Philadelphia.

Russell, D.W. and Setchell, K.D.R. 1992. Bile acid biosynthesis. *Biochemistry* 31:4737-4749. doi: 10.1021/bi00135a001.

Temel, R.E. and Brown, J.M. 2012. Biliary and nonbiliary contributions to reverse cholesterol transport. *Curr. Opin. Lipidol.* 23:85-90. doi: 10.1097/MOL.0b013e3283508c21.

van der Veen, J.N., Kruit, J.K., Havinga, R., Baller, J.F.W., Chimini, G., Lestavel, S., Staels, B., Groot, P.H.E., Groen, A.K., and Kuipers, F. 2005. Reduced cholesterol absorption upon PPARδ activation coincides with decreased intestinal expression of NPC1L1. *J. Lipid Res.* 46:526-534. doi: 10.1194/jlr.M400400-JLR200.

van der Veen, J.N., van Dijk, T.H., Vrins, C.L.J., van Meer, H., Havinga, R., Bijsterveld, K., Tietge, U.J.F., Groen, A.K., and Kuipers, F. 2009. Activation of the liver X receptor stimulates trans-intestinal excretion of plasma cholesterol. *J. Biol. Chem.* 284:19211-19219. doi: 10.1074/jbc.M109.014860.

van der Velde, A.E., Vrins, C.L.J., van den Oever, K., Kunne, C., Oude Elferink, R.P.J., Kuipers, F., and Groen, A.K. 2007. Direct intestinal cholesterol secretion contributes significantly to total fecal neutral sterol excretion in mice. *Gastroenterology* 133:967-975. doi: 10.1053/j.gastro.2007.06.019.

van der Velde, A.E., Brufau, G., and Groen, A.K. 2010. Transintestinal cholesterol efflux. *Curr. Opin. Lipidol.* 21. doi: 10.1097/MOL.0b013e3283395e45.

van der Wulp, M.Y.M., Cuperus, F.J.C., Stellaard, F., van Dijk, T.H., Dekker, J., Rings, E.H.H.M., Groen, A.K., and Verkade, H.J. 2012a. Laxative treatment with polyethylene glycol does not affect lipid absorption in rats. *J. Pediatr. Gastroenterol. Nutr.* 55:457-462. doi: 10.1097/MPG.0b013e3182555ba9.

van der Wulp, M.Y., Lukovac, S., van Dijk, T.H., Bloks, V.W., Boekschoten, M.V., Dekker, J., Rings, E.H., Groen, A.K., and Verkade, H.J. 2012b. Transintestinal cholesterol excretion can be manipulated by dietary fat composition in mice. Available at *http://www.rug.nl/research/portal/files/14431496/04_c4.pdf!null*.

van Dijk, T.H. 2010. Computational analysis of carbohydrate metabolism: Stable isotope techniques in small laboratory animals. Doctoral thesis, Rijksuniversiteit Groningen.

van Dijk, T.H., Boer, T.S., Havinga, R., Stellaard, F., Kuipers, F., and Reijngoud, D.-J. 2003. Quantification of hepatic carbohydrate metabolism in conscious mice using serial blood and urine spots. *Anal. Biochem.* 322:1-13. doi: 10.1016/j.ab.2003.07.008.

van Dijk, T.H., Laskewitz, A.J., Grefhorst, A., Boer, T.S., Bloks, V.W., Kuipers, F., Groen, A.K., and Reijngoud, D.J. 2013. A novel approach to monitor glucose metabolism using stable isotopically labelled glucose in longitudinal studies in mice. *Lab. Anim.* 47:79-88. doi: 10.1177/0023677212473714.

Vrieze, A., Out, C., Fuentes, S., Jonker, L., Reuling, I., Kootte, R.S., van Nood, E., Holleman, F., Knaapen, M., Romijn, J.A., Soeters, M.R., Blaak, E.E., Dallinga-Thie, G.M., Reijnders, D., Ackermans, M.T., Serlie, M.J., Knop, F.K., Holst, J.J., van der Ley, C., Kema, I.P., Zoetendal, E.G., de Vos, W.M., Hoekstra, J.B.L., Stroes, E.S., Groen, A.K., and Nieuwdorp, M. 2014. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. *J. Hepatol.* 60:824-831. doi: 10.1016/j.jhep.2013.11.034.