

In vivo D₂O labeling to quantify static and dynamic changes in cholesterol and cholesterol esters by high resolution LC/MS[§]

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Abstract High resolution LC/MS-MS and LC/APPI-MS methods have been established for the quantitation of flux in the turnover of cholesterol and cholesterol ester. Attention was directed toward quantifying the monoisotopic mass (M₀) and that of the singly deuterated labeled (M+1) isotope. A good degree of isotopic dynamic range has been achieved by LC/MS-MS ranging from 3-4 orders of magnitude. Correlation between the linearity of GC/MS and LC atmospheric pressure photoionization (APPI)-MS are complimentary ($r^2 = 0.9409$). To prove the viability of this particular approach, male C57Bl/6 mice on either a high carbohydrate (HC) or a high fat (HF) diet were treated with ²H₂O for 96 h. Gene expression analysis showed an increase in the activity of stearoyl-CoA desaturase (Scd1) in the HC diet up to 69-fold ($P < 0.0008$) compared with the HF diet. This result was supported by the quantitative flux measurement of the isotopic incorporation of ²H into the respective cholesterol and cholesterol ester (CE) pools. We concluded that it is possible to readily obtain static and dynamic measurement of cholesterol and CEs in vivo by coupling novel LC/MS methods with stable isotope-based protocols.—Castro-Perez, J., S. F. Previs, D. G. McLaren, V. Shah, K. Herath, G. Bhat, D. G. Johns, S-P. Wang, L. Mitnaul, K. Jensen, R. Vreeken, T. Hankemeier, T. P. Roddy, and B. K. Hubbard. **In vivo labeling to quantify static and dynamic changes in cholesterol and cholesterol esters by high resolution LC/MS.** *J. Lipid Res.* 2011. 52: 159–169.

Supplementary key words flux • liquid chromatography-mass spectrometry • lipids

The liver plays a vital role in cholesterol metabolism (1–5) and homeostasis. In addition to this, production of bile acids from cholesterol also plays an important role in the secretion and degradation of plasma lipoproteins. A

high level of cholesterol in the body circulation is strongly associated with atherosclerosis (6–10). The source of cholesterol comes from different areas: dietary, de novo synthesis, and synthesis in extrahepatic tissues. The liver acts as a cross-junction at which cholesterol is incorporated into HDL/LDL, and then secreted as free cholesterol in the bile or in the form of bile salts/acids.

Studies of cholesterol metabolism typically require measurements of static concentrations of cholesterol to identify differences between models or to determine the presence or absence of a disease phenotype. The simultaneous use of stable or radio isotope flux analysis can aid in understanding the nature of a metabolic abnormality and yield information regarding the dynamics that contribute to altered or perturbed homeostasis.

Questions surrounding cholesterol dynamics have been addressed using isotopic-labeled water for nearly 70 years, beginning with the pioneering studies of Schoenheimer (11–13) based on the use of ²H₂O and the classical work of Dietschy (14–20) et al. based on ³H₂O. Considering the dose of radiation (typically in mCi) and the advances in mass spectrometry and related instrumentation (e.g., coupling to GC), it is not surprising that many investigators have turned their attention toward the use of the stable isotope ²H₂O. The elegant work on human sterol and fatty acid (FA) flux carried out by Schoeller et al. (21), Wong et al. (22, 23), and Jones et al. (24, 25) has opened up the

Abbreviations: APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; CE, cholesterol ester; CID, collision-induced dissociation; HC, high carbohydrate; HF, high fat; IRMS, GC-isotope ratio mass spectrometry; PL, phospholipid; Scd1, stearoyl-CoA desaturase; SREBP, sterol-regulatory element binding protein; TOF-MS, time of flight mass spectrometry; TG, triglyceride.

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number of applications for the use of deuterated water in flux lipid experiments.

Although the ability to couple static and dynamic measurements in studies of cholesterol metabolism is of obvious importance, most studies have been done under relatively low-level analytical resolution. For example, investigators typically examine the metabolism of free cholesterol and/or total cholesterol esters (CE). In addition, the classical GC/MS and GC-isotope ratio mass spectrometry (IRMS) methods are not suitable for routine use in high-throughput analyses because cholesterol esters are generally separated offline as a single pool (e.g., using TLC), and then subjected to saponification, extraction, derivatization, and finally mass spectrometry analysis. This is a more time consuming and laborious process, where given today's demands, especially in the discovery arena, it is essential to make decisions more quickly than ever.

Recent advances in high-resolution LC-MS/MS have enabled the rapid high-throughput analyses of complex mixtures, which can be used to obtain information regarding different lipid classes and subclasses. These instruments are able to analyze complex biological mixtures with minimal sample preparation.

A variety of different ionization techniques were utilized for the experiments described here. For example, cholesterol is particularly difficult to ionize by electrospray (ESI) mass spectrometry as its proton affinity is relatively low; however, ESI is suitable for measuring the ammoniated adduct of CE with good sensitivity. This finding is highlighted by a comparison of equimolar concentrations (1 $\mu\text{g}/\text{ml}$) of CE 16:0 and CE 18:0 analyzed by ESI and atmospheric pressure chemical ionization (APCI) (supplementary Fig. 1). For both saturated CEs, the ESI technique proved to have a better signal intensity than APCI mode (~ 7 - to 13-fold better). Having said that, other researchers, including Butovich et al. (26, 27), have shown the application of APCI to measure in one analytical run free cholesterol and cholesterol esters coupled to a reversed phase column. But in their research, they were not measuring metabolic flux of free cholesterol and cholesterol esters in plasma. In our study, obtaining the best possible sensitivity was key to measuring the M1/M0 isotopomer ratio for free cholesterol and cholesterol esters. One ionization mode, such as APCI, was not sensitive enough to measure both analytes (free cholesterol and cholesterol esters) in one analytical run.

In addition to the measurement of CEs by ESI, we investigated the use of atmospheric pressure photoionization (APPI) for the measurement of free cholesterol. The major advantage of APPI over APCI is that, for free cholesterol, we have experienced better limits of detection (between 3- and 4-fold better signal) than APCI mode (supplementary Fig. II). This technique has been used in the past to ionize less polar biochemicals, such as sterols and steroids (28–34). The ionization mechanism is somewhat different from electrospray because in most cases a dopant is used to provide the proton to complete the ionization process. Typical dopants that may be used are acetone and toluene. The ionization is initiated by 10-eV photons emitted by a krypton discharge lamp. The mechanism of ionization by APPI involves the ab-

sorption of photons by the molecule(s) to be analyzed, followed by the ejection of an electron resulting in a molecular cation M^+ . This reaction only occurs if the ionization energy of the dopant is lower than the ionization energy of the photons. The dopant provides the proton, and the radical cation will react with the dopant to form a stable $[M+H]^+$ cation.

We report here on a simple method(s) for dissecting cholesterol metabolism via LC-MS/MS. We aimed to determine if we could simultaneously quantify the abundance and the isotopic labeling following $^2\text{H}_2\text{O}$ administration (35, 36) of different CE species in the presence of a high-carbohydrate (HC) or high-fat (HF) diet.

MATERIALS AND METHODS

Mice and diets

Male C57Bl/6 mice from Taconic were acclimated in the animal facility for one week. At an age of 10-weeks-old, mice were randomized into two groups ($n = 26$ per group), and the diet was switched to either a high carbohydrate (HC) diet (D12450, 10% fat, 70% carbohydrate, and 20% protein, Research Diets, NJ) or a carbohydrate-free (CF) diet (D12369B, 90% fat, 0% carbohydrate, and 10% protein, Research Diets, NJ). The diet intervention proceeded for 13 days, and then all mice were then given an intraperitoneal injection of labeled water (20 ml/kg of body weight, 99% $^2\text{H}_2\text{O}$). After injection, mice were returned to their cages ($n = 6$ mice per cage) and maintained on 5% ^2H -labeled drinking water for the remainder of the study. This design is sufficient to maintain a steady-state ^2H labeling of body water. Mice in each group were fed the respective diets ad libitum. They were sedated on various days after injection ($n = 6$ per day per group), blood and tissue samples (liver tissue was used for gene expression analysis only) were then collected and quick-frozen in liquid nitrogen. Samples were stored at -80°C until analyzed. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories (Rahway, NJ).

Water labeling

The ^2H -labeling of plasma water was determined as described by Shah et al. (37). Briefly, ^2H present in water is exchanged with hydrogen bound to acetone by incubating samples (e.g., 10 μl of plasma or known standards) in a 2 ml glass screw-top GC vial at room temperature for 4 h with 2 μl 10N NaOH and 5 μl of acetone. The instrument is programmed to inject 5 μl of headspace gas from the GC vial in a splitless mode. Samples were analyzed using a 0.8 min isothermal run (Agilent 5973 MS coupled to a 6890 GC oven-fitted with a DB-17 MS column, 15 m \times 250 μm \times 0.15 μm ; the oven was set at 170°C ; and helium carrier flow was set at 1.0 ml \times min $^{-1}$), acetone eluted at ~ 0.4 min, and the mass spectrometer was set to perform selected ion monitoring of m/z 58 and 59 (10 ms dwell time per ion) in the electron impact ionization mode.

GC/MS of total plasma palmitate and cholesterol

Plasma samples for GC/MS analysis were processed in 1.5 ml eppendorff tubes. An amount of 25 μl internal standard (FA 17:0, 0.5 mg/ml CHCl_3) and 100 μl 1N KOH in 80% ethanol was added to 50 μl of plasma. The samples were heated at 65°C for 1 h. Samples were acidified with 25 μl 6N HCl and extracted in 125 μl chloroform followed by vigorous vortexing for 20 s. The samples were centrifuged at 3000 rpm for 5 min, and then 100 μl of chloroform (lower layer) was collected and evaporated to dryness under N_2 .

Samples were derivatized using bis trimethyl silyl trifluoroacetamide (BSTFA) plus 10% trimethylchlorosilane (TMCS), 50 μ l was added to the sample, and then it was incubated at 75°C for 1 h. Excess BSTFA was evaporated to dryness in N_2 . The TMS derivative was reconstituted in 50 μ l ethyl acetate for analysis by GC/MS.

Samples were analyzed by GC/MS using the Agilent 6890 gas chromatograph linked to an Agilent 5973 mass selective detector (MSD) (Agilent, Palo Alto, CA) operated at 70 eV. Gas chromatography was performed using an Agilent J and W DB-5MS capillary column (30.0 m \times 250 μ m \times 0.25 μ m). An amount of 2 μ l was injected in a 20:1 split. The inlet temperature was set at 250°C and the helium gas carrier flow was set at 1 ml/min⁻¹. The oven temperature was started at 150°C, raised at 20°C per min to 310°C, and held at this temperature for 6 min.

The MSD was set for selected ion monitoring (SIM) of m/z 313, 314 for the palmitate TMS derivative; 327, 328, 329 for heptadecanoic acid TMS derivative; and 368, 369 for cholesterol TMS derivative with 10 ms dwell time per ion. Concentrations of fatty acids/cholesterol were corrected for by a standard curve with varying combinations of fatty acid or cholesterol with their respective D1-derivatives.

LC/MS of lipids

Plasma samples from each animal (20 μ l) were extracted for lipid analysis by LC/MS-MS using a dichloromethane (DCM)/methanol mixture (2:1, v/v) in accordance with the method described by Bligh and Dyer (38, 39). During the procedure, the samples were spiked with non-naturally occurring and deuterated lipid internal standards (17:0 containing CE and D6-cholesterol; Sigma Aldrich, St Louis, MO) in final concentrations of 2 μ g/ml.

The inlet system was composed of an Acquity UPLC (Waters, Milford, MA). Mouse plasma lipid extracts were injected (10 μ l) onto a 1.8 μ m particle 100 \times 2.1 mm id Waters Acquity HSS T3 column (Waters); the column was maintained at 55°C. The flow rate used for these experiments was 0.4 ml/min. A binary gradient system consisting of acetonitrile (Burdick and Jackson, USA) and water with 10 mM ammonium formate (Sigma-Aldrich) (40:60, v/v) was used as eluent A. Eluent B consisted of acetonitrile and isopropanol (Burdick and Jackson) containing 10 mM ammonium formate (10:90, v/v). The sample analysis was performed by using a linear gradient (curve 6) over a 15 min total run time. During the initial portion of the gradient, it was held at 60% A and 40% B. For the next 10 min, the gradient was ramped in a linear fashion to 100% B and held at this composition for 2 min. Then the system was switched back to 60% B and 40% A and equilibrated for an additional 3 min. For the free cholesterol measurements by LC/APPI-MS, the gradient conditions were identical apart from the fact that no ammonium formate was used as the additive.

The inlet system was directly coupled to a hybrid quadrupole orthogonal time-of-flight mass spectrometer (SYNAPT G2 HDMS, Waters, MS Technologies, Manchester, UK). Electrospray positive and APPI positive ionization modes were used. In ESI mode, a capillary voltage and cone voltage of ± 2 kV and ± 30 V, respectively, was used. The desolvation source conditions were as follows: for the desolvation gas, 700 l/hr was used and the desolvation temperature was kept at 450 C. APPI was utilized using a krypton discharge lamp (10-eV photons) set with a repeller voltage of ± 3.5 kV. The dopant used for the APPI experiments was acetone (Fisher Scientific, Pittsburgh, PA), which was infused at a continuous flow rate of 100 μ l/min post column. The desolvation source conditions for APPI were as follows: for the desolvation gas, 900 l/hr was used and the desolvation temperature was kept at 600 C. Data were acquired over the mass range of 50-1200 Da for both MS and MS^E modes (40-43). The mass spectral resolution was set to 25K full width half mass (FWHM) and typical mass accuracies were in the order of 0-2 ppm. The system was equipped

with an integral LockSpray unit with its own reference sprayer that was controlled automatically by the acquisition software to collect a reference scan every 10 s lasting 0.3 s. The LockSpray internal reference used for these experiments was Leucine enkephalin (Sigma-Aldrich) at a concentration of 5 ng/ μ l in 50% acetonitrile/50% H₂O plus 0.1% formic acid (v/v). The reference internal calibrant was introduced into the lock mass sprayer at a constant flow rate of 50 μ l/min using an integrated solvent delivery pump. A single lock mass calibration at m/z 556.2771 in positive ion mode was used during analysis. The mass spectrometer was operated in the MS^E mode of acquisition. During this acquisition method, the first quadrupole Q1 is operated in a wide band RF mode only, allowing all ions to enter the T-wave collision cell. Two discrete and independent interleaved acquisitions functions are automatically created. The first function, typically set at 5 eV, collects low energy or unfragmented data, and the second function collects high energy or fragmented data typically set by using a collision energy ramp from 25-35 eV. In both instances, argon gas is used for collision-induced dissociation (CID). This mode of operation allows for fragmentation ions to be generated ad hoc, and the use of the software data mining tool allowed for the alignment of the low and high energy data. This mode of acquisition proved to be adequate for high throughput screening, but there are some cases where complete ion co-elution occurs between the low and high energy acquisitions. When this occurred, more rigorous fragmentation MS/MS CID experiments were utilized.

Data processing and statistical analysis

For the LC/MS and GC/MS analysis of the isotopic dilution of cholesterol, a set of standards were prepared in chloroform (1 mg/ml), ranging from 0% excess ²H-labeling up to 2.5% excess ²H-labeling (0%, 0.15%, 0.3%, 0.6%, 1.25%, 2.5%) by mixing with cholesterol and its D1-derivative. Labeled palmitate was prepared in the same fashion as cholesterol for the GC/MS analysis only, and heptadecanoic acid was used as the internal standard. All the samples were diluted 10-fold with (65:5:30 v/v/v) IPA:MeOH:H₂O to achieve a final concentration of 0.1 mg/ml. For GC/MS analysis 10 μ l of each standard was derivatized with BSTFA as described above. For the quantitation of the contribution of cholesterol synthesis, the data was processed using a precursor:product labeling ratio to the general equation: % newly made material = product labeling / (water labeling \times n) \times 100, where n is the number of exchangeable hydrogen, assumed to equal 26, and the product labeling is determined from the ratio of M1/M0 isotopomers (44).

The GC/MS and LC/MS data acquired were processed by the instrument manufacturer's software (ChemStation and MassLynx, respectively). Gene expression data were processed using Ingenuity software (Ingenuity Systems, Redwood City, CA). For statistical analysis, all data are presented as \pm SEM. Differences between groups were computed by Student's *t*-test (GraphPad Prism, La Jolla, CA). Post-test analysis for quantifiable variables was conducted using Mann-Whitney *U* nonparametric test with two-tailed *P* values. *P* < 0.05 was considered statistically significant for all data derived from the experiments.

RNA isolation and real-time quantitative PCR analysis

Liver tissue (\sim 20 mg) was snap-frozen in liquid nitrogen and stored at -80°C . The tissues were homogenized in 600 μ l RLT lysis buffer (Qiagen, Valencia, CA) containing 0.1% (v/v) β -mercaptoethanol using a PowerGen 125 homogenizer and 7 \times 65 ml disposable plastic generators (Fisher Scientific). Total RNA was extracted from the homogenized tissue using RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. cDNA was generated from 2 μ g of RNA using RT² First Strand

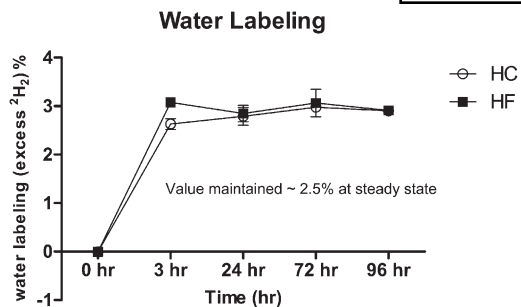


Fig. 1. Steady-state labeling of plasma water in mice fed with a HF and HC diets. HC, high carbohydrate; HF, high fat.

kit (SA Biosciences). Real-time PCR analysis was performed on the 7900HT PCR System (Applied Biosystems, Foster City, CA) with 2× SYBR PCR Master Mix and mouse-specific PCR primers for mouse Scd1, (SABiosciences). Expression levels of stearoyl-CoA desaturase (Scd1) mRNA were normalized to an average of

that of mouse β-actin (Actb), glyceraldehyde 3-phosphate dehydrogenase (Gapdh), beta-glucuronidase (Gusb), hypoxanthine-guanine phosphoribosyltransferase (Hprt1), peptidylprolyl isomerase A (cyclophilin A) (Ppia), and ribosomal protein 113a (Rpl13a) in each sample.

RESULTS

The water labeling after 3 h was kept constant for both diets for the duration of the study at ~2.5% (**Fig. 1**).

Gene expression results from the HC diet revealed an upregulation in the expression of sterol-regulatory element binding protein (SREBP)1c pathway, which contain enzymes involved in de novo lipogenesis, including stearoyl-CoA desaturase (Scd1). In contrast, the SREBP2 pathway was downregulated. Expression of Scd1 was 69-fold higher ($P < 0.0008$) on the HC diet compared with the HF diet. There is substantial evidence in the literature

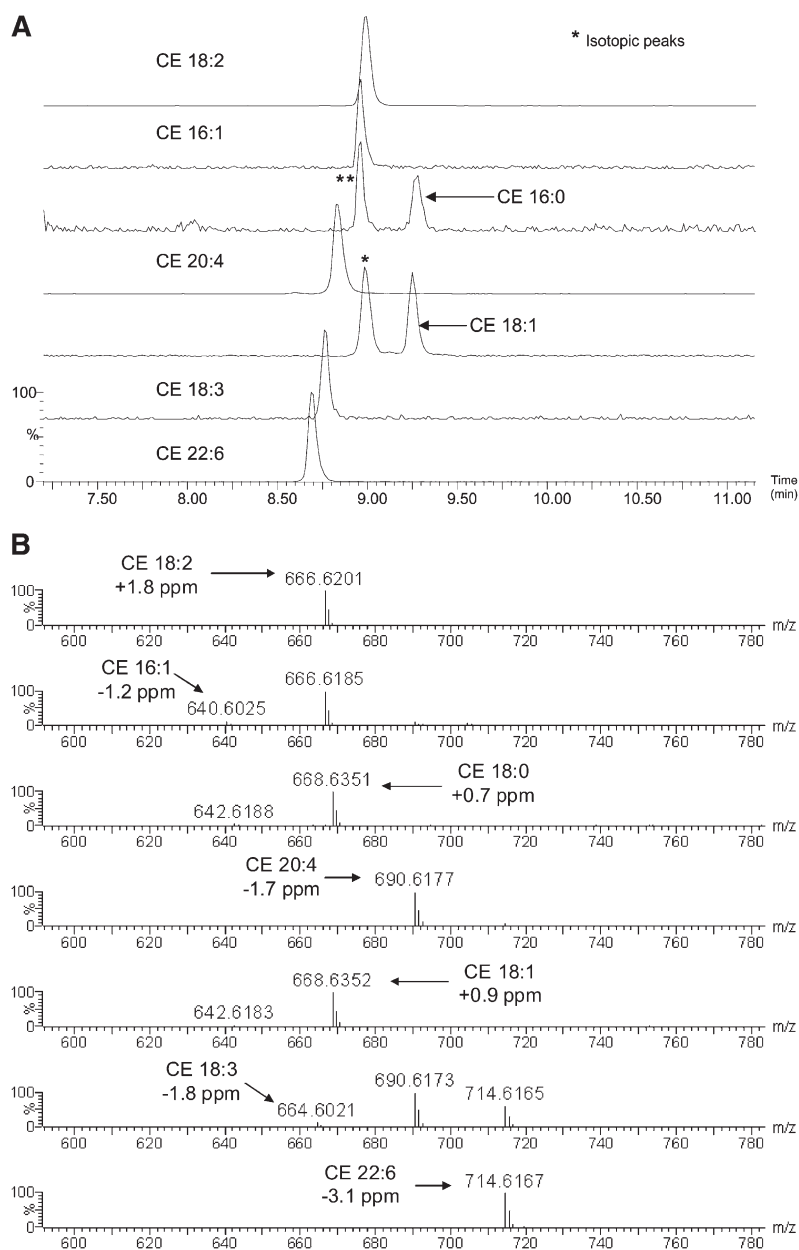


Fig. 2. A: LC/MS profiles of plasma CE by LC/ESI-TOF-MS. Data are shown as the extracted ion chromatogram for each ester. *M+2 isotopes of CE 18:2. **M+2 isotopes of CE 16:1. B: Full scan MS spectra with exact mass for the corresponding CE. †Note: cholesterol esters detected in electrospray positive ion mode as ammonium adducts. CE, cholesterol ester; TOF-MS, time of flight mass spectrometry.

TABLE 1. Isotopic dynamic range for different CE standards by LC/ESI-TOF-MS

CE Standard	Cholesterol Ester Concentration (μM)									
	0.01		0.1		1		10		100	
	M1/M0 <i>ratio</i>	CV <i>%</i>	M1/M0 <i>ratio</i>	CV <i>%</i>	M1/M0 <i>ratio</i>	CV <i>%</i>	M1/M0 <i>ratio</i>	CV <i>%</i>	M1/M0 <i>ratio</i>	CV <i>%</i>
CE 16:0	ND ^a	ND ^a	0.488 \pm 0.011	2.18	0.469 \pm 0.003	0.54	0.479 \pm 0.006	1.15	0.467 \pm 0.004	0.81
CE 16:1	ND ^a	ND ^a	0.475 \pm 0.01	2.03	0.473 \pm 0.009	1.89	0.473 \pm 0.012	2.43	0.478 \pm 0.004	0.73
CE 18:0	ND ^a	ND ^a	0.493 \pm 0.003	0.61	0.503 \pm 0.007	1.43	0.499 \pm 0.007	1.34	0.495 \pm 0.006	1.28
CE 18:1	ND ^a	ND ^a	0.499 \pm 0.011	2.14	0.499 \pm 0.007	1.42	0.506 \pm 0.001	0.2	0.506 \pm 0.002	0.3
CE 18:2	0.502 \pm 0.008	1.55	0.501 \pm 0.009	1.72	0.508 \pm 0.004	0.79	0.506 \pm 0.003	0.64	0.506 \pm 0.003	0.5

In all cases, a good level of precision was shown. The isotopic dynamic range was between 3 and 4 orders of magnitude. CE, cholesterol ester; CV, coefficient of variation; ND, not detected; TOF-MS, time of flight mass spectrometry.

^aCholesterol ester was not detected at this concentration.

(45–49) linking over-intake of carbohydrates to de novo lipogenesis (DNL). This is accompanied by the synthesis of FA and the corresponding incorporation into the different phospholipid (PL), triglyceride (TG) and CE pools. When Scd1 is induced, it results in increased desaturation rate of palmitate (FA 16:0) and stearate (FA 18:0) at the $\Delta 7$ and $\Delta 9$ position of the fatty acyl chain, respectively, to give rise to FA 16:1 $\Delta 7$ and FA 18:1 $\Delta 9$. These newly desaturated fatty acids serve as substrates for other enzymes to promote the synthesis of PL, TG, and CE. The lipogenic cholesterol ester index was calculated as the ratio of palmitic acid (FA 16:0)-containing CE and linoleic acid (FA 18:2 $\Delta 6$)-containing CE. This index was increased 2.7 times ($P = 0.0011$) in the HC diet versus the HF diet. External calibrations were used for the calculation of the CE 16:0 and CE 18:2 ratio as the mass spectral responses were different (supplementary Fig. III-A). Scd1 activity was derived by measuring the desaturation index (DI) between the ratio of palmitoleic acid (FA16:1 $\Delta 7$)-containing CE and palmitic acid (FA 16:0)-containing CE; for this measurement external calibrations were also utilized for CE 16:0 and CE 16:1 (supplementary Fig. III-B). As a result of this analysis, DI increased considerably in the HC diet (~ 3.6 -fold, $P = 0.0011$). In this study, the main focus was placed on the information that can be provided by LC-MS/MS compared with GC/MS for the quantifiable flux of both free cholesterol and CE measurements using an in vivo preclinical murine animal model. A rapid and robust analytical method was developed by LC/ESI-MS and LC/APPI-MS for CE and free cholesterol analysis. This LC/MS lipid method was not exclusively limited to free cholesterol and CE but to other lipids, such as FFA, LysoPL, PL, DG, SM, Cer, and TG. By the use of this LC/MS method, the following cholesterol esters in positive ion electrospray mode were detected as ammonium adducts $[\text{M}+\text{NH}_4]^+$ (Fig. 2A, B) in both HC and HF diets; CE 16:0, CE 16:1, CE 18:1, CE 18:2, CE 18:3, CE 20:4 and CE 22:6. Free cholesterol was detected in APPI positive ion mode. In order to prove the robustness of the analytical platform developed, different concentrations (0.01 μM , 0.1 μM , 1 μM , 10 μM , and 100 μM) of unlabeled cholesterol esters were analyzed

($n = 3$ for each concentration). The goal behind this experiment was to determine at which point in the titration curve the ratio between M1/M0 will become imprecise especially at low concentrations when ion statistics may be lower than at the top spectrum of the titration curve. As can be observed in Table 1, there was a good level of precision of measurements throughout all different concentrations for the CE utilized in this experiment. For the majority of cases, three orders of isotopic dynamic range were shown (0.1–100 μM); only in one instance, for CE 18:2, where four orders of isotopic dynamic range (0.01–100 μM) was achieved. The low concentration of the titration curve showed a higher degree of inaccuracy, but as previously mentioned, this is not an abnormal finding as the level of robust ion statistics is lower than at higher concentrations. Nevertheless, it was found to be acceptable for the type of measurements conducted in this study. When all concentrations belonging to the 18 and 16 FA acyl-containing cholesterol esters were combined together (Fig. 3), coefficients of variation of 1.33% ($n = 38$) and 1.54% ($n = 22$) were achieved, respectively. Therefore, irrespective of the concentration of the analyte in the biological matrix, good

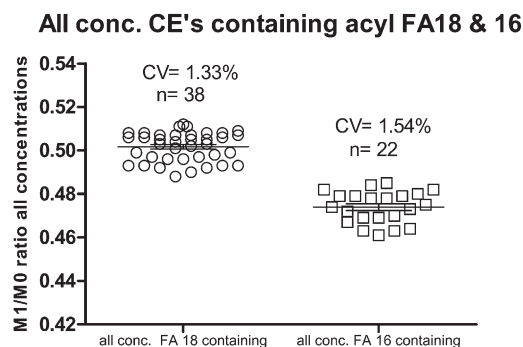


Fig. 3. M1/M0 ratio for all concentrations containing acyl FA 18 or 16 for the CEs analyzed. The combination of all concentrations (0.01–100 μM) for the acyl FA 18-containing CEs showed good coefficient of variation 1.33% ($n = 38$). The combination of all concentrations (0.1–100 μM) for the acyl FA 16-containing CEs showed good coefficient of variation 1.54% ($n = 22$). CE, cholesterol ester.

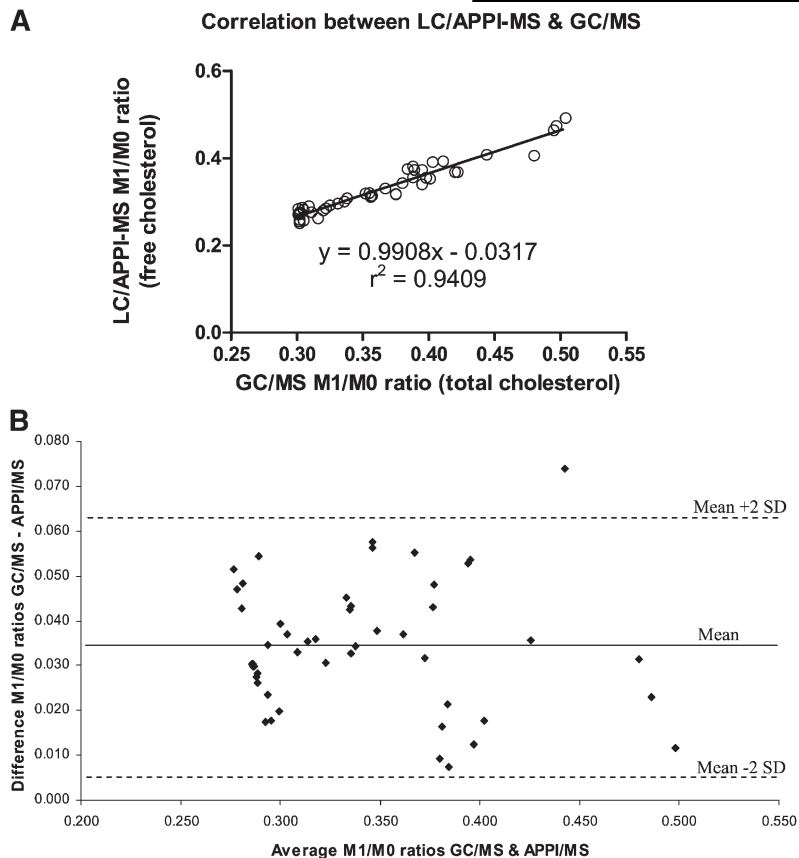


Fig. 4. Correlation and isotopic enrichment by ^2H between GC/MS (total cholesterol) and LC/APPI-MS (free cholesterol) in the presence of HC/HF diets. A: Linearity of the isotopic enrichment by GC/MS and LC/APPI-MS. B: Differences between GC/MS and LC/APPI-MS against the mean of the two measurements for each individual data set, showing the mean difference and upper and lower 95% limits of agreement (mean + 2SD and mean - 2SD). APPI, atmospheric pressure photoionization; HC, high carbohydrate; HF, high fat.

levels of precision were observed at a wide isotopic dynamic range between three and four orders as described here. This level of dynamic range was not previously achievable with TOF-MS, but new developments in ion detection (e.g., analog to digital converter) have allowed the application of this tool for flux analysis. This is a relevant observation as the levels of endogenous CE and/or free cholesterol will vary throughout different samples and studies.

Historically GC/MS and GC/IRMS have been the analytical platforms of choice, and they have shown a good linearity when isotopic dilutions are conducted. In fact,

the use of isotopic dilution is very important because the titration curve is used to read the isotopic enhancement from a particular $^2\text{H}_2\text{O}$ in vitro or in vivo experiment. Therefore, for the current study, it was important to examine the correlation between the two analytical platforms for isotopic dilution titration curves. A range of different isotopic dilutions ranging from 0% excess ^2H -labeling up to 2.4% excess ^2H -labeling of cholesterol was prepared by mixing with unlabeled cholesterol and [$3\text{-}^2\text{H}$] cholesterol. **Fig. 4A** describes very clearly the correlation between LC/APPI-MS and GC/MS measurements

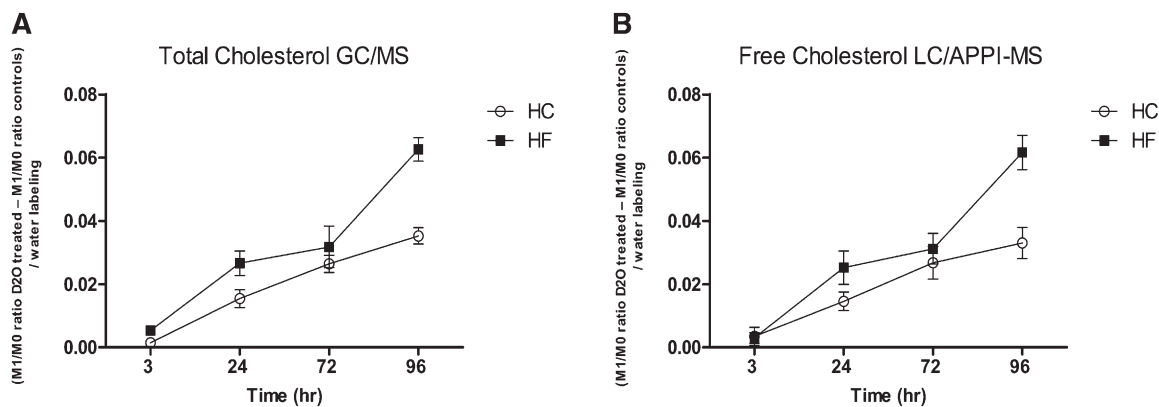


Fig. 5. Linearity and correlation of total cholesterol GC/MS and free cholesterol LC/APPI-MS used in the context of a biological study. A: Total cholesterol ^2H incorporation for HC and HF diets. B: Free cholesterol ^2H incorporation for HC and HF diets. In both scenarios, there is a higher incorporation of the ^2H label in the HF diet versus the HC diet. APPI, atmospheric pressure photoionization; HC, high carbohydrate; HF, high fat.

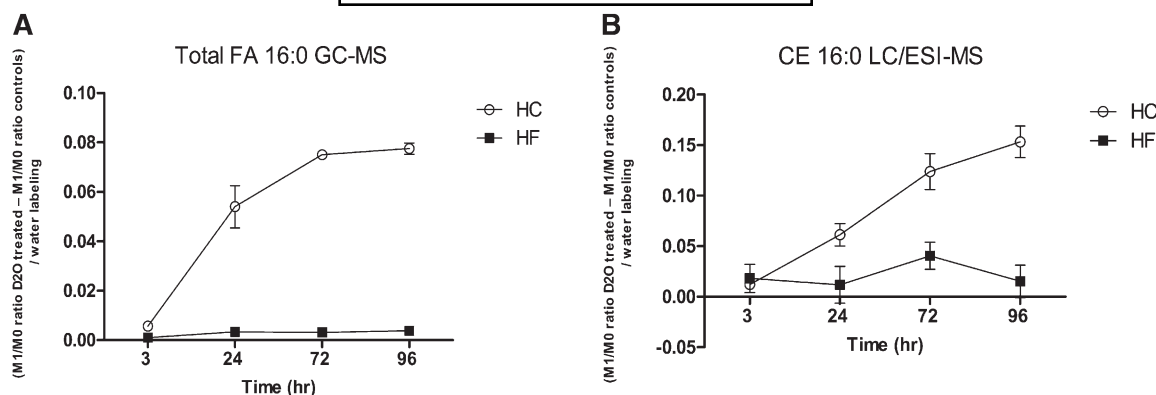


Fig. 6. Isotopic labeling correlation in the measurement of total palmitate in plasma by GC/MS versus the measurement of CE 16:0 in plasma by LC/ESI-MS. A: Indicates that palmitate de novo synthesis in the HC diet is very substantial. B: Describes more selective and complimentary tools in which it is possible to measure the level of incorporation of the labeled palmitate in the CE pool. CE, cholesterol ester; HC, high carbohydrate; HF, high fat.

for the deuterium isotopic enrichment of free cholesterol (LC/APPI-MS) and total cholesterol (GC/MS), respectively, from the different diets. The plot contains all of the data in the study. A very good correlation factor was achieved ($r^2 = 0.9409$ with best-fit values of slope and intercept $y = 0.9908$ and $x + 0.0317$). Note that for the GC/MS measurements, the isotopic enrichment in a normal setting would represent the total cholesterol measurement, while for the LC/APPI-MS measurements, more specificity can be achieved by this analytical platform without further sample preparation (i.e., the samples do not need to be saponified), and free cholesterol can be differentiated from CE. Even though we achieved a good level of linearity between the two measurements by the different analytical techniques, Bland-Altman et al. (50) suggested that a more statistically relevant approach to measure this correlation is by measuring the agreement between the two techniques. The method of measuring agreement is based upon the computation of the mean differences between the two techniques and the likelihood that the data agrees with the 95% confidence limits set by the mean +2SD and the mean -2SD. This measurement can be observed in Fig. 4B. A good degree of agreement was shown between the two methods, as most of the results apart from one outlier fell outside the mean +2SD and mean -2SD confidence limits.

Measurement of ^2H enhancement can be realized by using this technique, but there are certain limitations that need to be noted. If the level of enrichment is below 0.3%, then other techniques, such as IRMS, would be more

appropriate. Nonetheless, IRMS provides total incorporation of deuterated label but specific lipid enrichment by deuterium is lost.

Having proved the ruggedness and the linearity of this new analytical platform, it was necessary to test this approach in the context of biology and with the use of $^2\text{H}_2\text{O}$ for an in vivo study. The first point of interest was to determine whether it was possible to achieve a level of correlation between the two techniques from the biological samples already described in detail in "Materials and Methods." Therefore, the first step was to analyze the samples for free cholesterol content. It is clear that GC/MS (Fig. 5A) will only provide information about the total cholesterol labeling in contrast to LC/APPI-MS, which is more selective (Fig. 5B) and provides information about the free cholesterol labeling measurement. The important point to note here is that the isotopic enhancement detected by either analytical platform is identical. Therefore, these results showed good correlation with other analytical techniques such as GC/MS. Next, total palmitate was reported by GC/MS (Fig. 6A), and when compared to the CE 16:0 LC/MS method (Fig. 6B), it showed a very similar trend in both cases; mainly, deuterium label incorporation was increased by the HC diet instead of the HF diet. In the case of the measurements made by LC/MS, we now have the capability to monitor individual lipid pools rather than observing/measuring incorporation in the total FA pools.

To achieve an even higher degree of selectivity, CE found by this method were further subjected to CID

TABLE 2. Percentage of newly synthesized cholesterol per hour for HC and HF diets

Diet	Cholesterol Ester ^a							Average \pm SD
	16:0	16:1	18:1	18:2	18:3	20:4	22:6	
	%	%	%	%	%	%	%	%
High carbohydrate	0.031	0.03	0.027	0.033	0.038	0.029	0.03	0.031 \pm 0.004
High fat	0.053	0.045	0.052	0.052	0.056	0.05	0.05	0.051 \pm 0.003

Percentage of newly synthesized cholesterol per hour measured in plasma by LC/ESI-TOF-MS for the HC and HF diets. HC, high carbohydrate; HF, high fat; TOF-MS, time of flight mass spectrometry.

^aCholesterol esters were fragmented by LC/MS-MS to provide the cholesteryl motif.

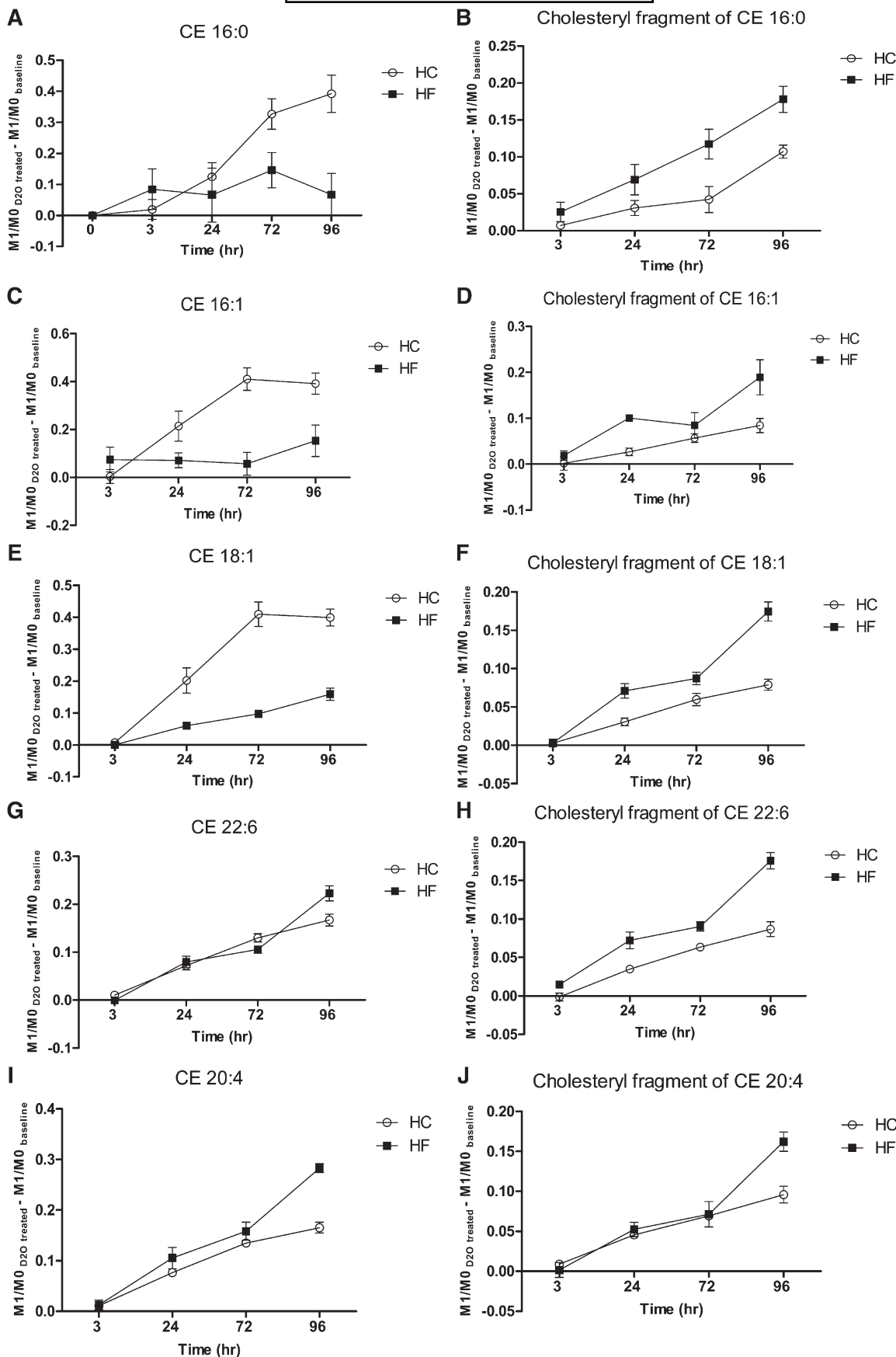



Fig. 7. Measurement of ^2H isotope labeling for CEs and cholesteryl motif in plasma by LC/ESI-MS/MS for CE16:0, CE 16:1, CE 18:1, CE 22:6, and CE 20:4. By observing the M1/M0 ratios, (A) shows that palmitate is driving the synthesis in the HC diet, while the opposite is true for (B) where the cholesteryl motif shows that the ^2H incorporation mainly takes place in the HF diet. This event repeats itself in (C).

experiments to obtain the cholesteryl motif fragment ion by electrospray mass spectrometry. This was necessary to determine whether the level of FA synthesis or cholesterol synthesis was responsible for the ^2H label incorporation in the CE in question. For both diets, the following CE were detected: CE 16:0, CE 16:1, CE 18:1, CE 18:2, CE 18:3, CE 20:4, and CE 22:6. The data in **Table 2** show that the newly synthesized cholesterol in the HF diet (0.051% newly synthesized cholesterol per h \pm 0.003 SD) was higher than that in the HC diet (0.031% newly synthesized cholesterol per h \pm 0.004 SD). In **Fig. 7**, it can be divided in several portions. **Fig. 7A** shows how the palmitate is actually driving the incorporation of ^2H in CE 16:0 for the HC diet. This is not an unusual finding to observe because this particular diet contains a high degree of palmitate and stearate (18.45% and 8.48% by FA individual content HC diet, analyzed by GC-FID). This is very well correlated with the findings in **Fig. 7C** and **7E**, where there is an increment in the level of palmitate and stearate in the HC diet. *Scd1* is induced and therefore a similar trend should be expected here for the CE 16:1 and CE 18:1. Interestingly enough, when CE 16:0, CE 16:1, and CE 18:1 are all subjected to CID fragmentation to release the cholesteryl motif (supplementary Fig. IV), it can be noted that the trend is now reversed (**Fig. 7B, D, F**), and the cholesterol in the HF diet is driving the level of incorporation of the ^2H label in the cholesterol pool either from diet or de novo synthesis. For the PUFA, the cholesterol ester moieties trend was somewhat reversed to what was observed with the saturated and monounsaturated FA in the cholesterol ester motif. In **Fig. 7H** and **7J**, it can be seen that the cholesterol motif is clearly driving the level of incorporation of the ^2H label for CE 20:4 and CE 22:6 for the HF diet. This may point to the fact that the pool of the PUFA available is relatively smaller in the case of the HC diet, and therefore, the turnover in the FA pool is smaller in comparison with the size of the cholesterol pool in the HF diet.

DISCUSSION

We have demonstrated a fast and robust method of analysis for quantifying the flux of cholesterol and cholesterol esters *in vivo*. By this approach, it is possible to obtain quantitative static and dynamic information not only for sterols but also for another lipid classes. The combination of atmospheric pressure ionization CID mass spectrometry with high resolution allowed us to obtain a high degree of specificity from a diet intervention study (HC/HF diets) to determine the predominant incorporation of the deuterium label present, preferably in the cholesteryl motif or the fatty acyl chain. This level of specificity is the major advantage over other techniques, such as IRMS, which requires much more sample preparation to obtain this detailed degree of information.

Even though IRMS is the technique of choice for isotopic enrichment measurements we have proven that for levels > 0.3 atom % excess enrichment deuterium labeling, it is not necessary to obtain the very low levels of isotopic detection and precision offered by IRMS. This is certainly the case for most studies conducted in the pre-clinical rodent models, as higher dosage levels of deuterated water may be used as described in this study. In humans and nonhuman primates, the deuterated water administered for flux studies results in a much lower deuterium isotope enrichment ~ 0.05 atom % excess (25); therefore, it would be more appropriate to utilize IRMS. In our experience this is certainly the case, and very informative data can be generated in this way. Measuring dynamic changes not only for sterol biochemistry but also for other lipid metabolites proves to be a very powerful tool for cardiovascular research, as it will allow focusing on the rate of de novo lipid synthesis. Another important aspect for this approach is that the use deuterated water at safe levels does not perturb metabolic system homeostasis.

A total integrated approach was highlighted in this study, which consisted of detection of genes and enzymes that are upregulated or downregulated by a biological insult. These genes will promote or signal a specific enzyme or enzymes to act upon the induction or inhibition of a single or multiple metabolic pathway(s). The measurements of these final biological endpoints by themselves have little significance if there is no traceability to the source(s). In addition, static measurement of lipids does not provide the level of specificity that can be generated by flux analysis. For instance, *Scd1*, which is an enzyme involved in de novo lipogenesis, was markedly upregulated in the HC diet, and as a consequence, there was an increase in the amount of the deuterium label incorporated in the palmitate and stearate FA pools, which in turn led to the conversion to palmitoleic and oleic acid. The administration of stable-isotope tracers *in vivo* coupled to LC/MS-MS provides a deeper insight regarding the dynamics of specific lipid classes. The advantage of this very selective approach to monitor and quantify individual lipid pools is that in one single experiment, an information-rich result in terms of quantitative and qualitative lipid composition may be achieved. Use of this analytical strategy will allow us to simplify analyzing samples and reporting analytical and biological data in a drug discovery setting. The combination of a platform composed of gene expression, which can guide us toward the biochemical pathway perturbed by genetic or novel pharmacological intervention, with a multiplexed platform, which enables us to monitor de novo synthesis and has the ability to measure qualitative and quantitative changes, proves to be a unique and very desirable platform, especially at the very early stages of drug discovery. 

through (F). In (G) through (J), it is significantly different from the rest because in both cases for the CE 22:6 and CE 20:4, the cholesterol seems to be driving the incorporation of ^2H primarily in the HF diet. CE, cholesterol ester; HC, high carbohydrate; HF, high fat.

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