Dynamic system-wide mass spectrometry based metabolomics approach for a new Era in drug research

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Chapter 8

In-vivo 'heavy water' labeling in C57Bl/6 mice to quantify static and kinetic changes in free cholesterol and cholesterol esters by LC/MS

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Part III: Chapter 8

In-vivo 'heavy water' labeling in C57Bl/6 mice to quantify static and kinetic changes in free cholesterol and cholesterol esters by LC/MS

SUMMARY

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 towards quantifying the monoisotopic mass (M0) and that of the singly-deuterated labeled (M+1) isotope. A good degree of isotopic dynamic range was achieved by LC/MS-MS ranging from 3-4 orders of magnitude. Correlation between the linearity of GC/MS and LC/APPI-MS are complimentary (r^2 =0.9409). In order 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 ^2H_2O for 96 hours. Gene expression analysis showed an increase in the activity of stearoyl-CoA desaturase (Scd1) in the HC diet up to x 69 fold (p <0.0008) in comparison 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 pools. From these results, it was concluded that it is possible to readily obtain static and dynamic measurement of cholesterol and cholesterol esters *in-vivo* by coupling novel LC/MS methods with stable isotope-based protocols.

INTRODUCTION

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 extra hepatic tissues. The liver acts as a cross-junction at which cholesterol is incorporated into HDL/LDL, and 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 yielding 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, with the pioneering studies of Schoenheimer (11-13) based upon the use of $^{2}H_2O$ and the classical work of Dietschy (14-20) and colleagues relied on $^{3}H_2O$. 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 towards the use of the stable isotope, $^{2}H_2O$. The human sterol and fatty acid flux elegant work carried out by Schoeller *et al* (21), Wong *et al* (22, 23), and Jones *et al* (24, 25) has opened up the 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 a relatively low level analytical resolution. For example, investigators typically examine the metabolism of free cholesterol and/or total cholesterol esters. In addition, the classical GC/MS and GC-Isotope Ratio Mass Spectrometry (IRMS) methods are not suitable for routine use in high-throughput analyses since cholesterol esters are generally separated off-line as a single pool (e.g. using TLC), and then subjected to saponification, extraction, derivatization and finally mass spectrometry analysis, this supposes a more time consuming and laborious process in which in today's new demands specially in the discovery arena time is of the essence to make decisions in a faster manner than ever before.

Recent advances in high resolution LC-MS/MS have enabled the rapid high-throughput analyses of complex mixtures, which therein can be used to obtain information regarding different lipid classes and sub-classes. 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 herein. For example cholesterol is particularly difficult to ionize by electrospray (ESI) mass spectrometry as its proton affinity is relatively low, but on the other hand ESI is suitable for measuring the ammoniated adduct of cholesterol ester (CE) with good sensitivity. This

finding is highlighted by a comparison of equimolar concentrations (1µg/mL) of CE 16:0 and CE 18:0 analyzed by ESI and APCI (supplementary figure 1). For both saturated CE's the ESI technique proved to have a better signal intensity than in APCI mode (~7-13 x fold better). Having said that, other researchers in this field Butovich *et al* (26, 27) have shown the application of atmospheric pressure chemical ionization (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 this research article it was essential to obtain the best sensitivity to measure 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 CE's 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 3-4 x fold better signal than in APCI mode (supplementary figure 2). 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 utilized to provide the proton to complete the ionization process. Typical dopants which may be used are either acetone or toluene. The ionization is initiated by 10-eV photons emitted by a krypton discharge lamp. The mechanism of ionization by APPI involves the absorption of photons by the molecule(s) to be analyzed; this is then 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 previously mentioned will react with the dopant to form a stable $[M+H]^+$ cation.

In this research a simple method(s) for dissecting cholesterol metabolism via LC-MS/MS is reported, attention was aimed at determining if we could simultaneously quantify the abundance and the isotopic labeling following ^2H_2O administration (35, 36) of different cholesterol ester species in the presence of a high carbohydrate and high fat diets.

MATERIALS AND METHODS

Biological

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; all mice were then given an intraperitoneal injection of labeled water (20ml/kg of body weight, 99% ^2H_2O). 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*, and 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 Merck Research Laboratories Institutional Animal Care and Use Committee (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 hours 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, 15m x 250µm x 0.15µm, the oven was set at 170 ^0C and helium carrier flow was set at 1.0 ml x min^{-1}), acetone elutes at ~ 0.4 min, 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: To 50 µL of plasma 25 µL internal standard was added (FA 17:0, 0.5 mg/ml $CHCl_3$) and 100 µL 1N KOH in 80% ethanol. The samples were heated at 65°C for 1 hour. Samples were acidified with 25 µL 6N HCl and extracted in 125 µL chloroform followed by vigorous vortexing for 20 sec. The samples were centrifuged at 3000 rpm for 5 min, 100 µL of chloroform (lower layer) was collected and evaporated to dryness under N_2.

Samples were derivatized using Bis Trimethyl Silyl Trifluoroacetamide (BSTFA) + 10% Trimethylchlorosilane (TMCS), 50 µL was added to the sample and then incubated at 75°C for 1 hour. Excess BSTFA was evaporated to dryness in N_2. The TMS-derivative was re-constituted in 50 µL 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&W DB-5MS capillary column 30.0 m x 250 um x 0.25 µm. 2 µL 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 /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 (20µL) 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 extraction procedure the samples were spiked with a final concentration of 2 µg/mL with non-naturally occurring and deuterated lipids internal standards [(17:0 containing CE and D_6-Cholesterol, (Sigma Aldrich, St Louis, MO))].

The inlet system was comprised of an Acquity UPLC (Waters, Milford, MA, USA). Mouse plasma lipid extracts were injected (10µL) onto a 1.8 µm particle 100 x 2.1 mm id Waters Acquity HSS T3 column (Waters, Milford, MA, USA); 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 & Jackson, USA) and water with 10 mM ammonium formate (Sigma-Aldrich, St Louis, MO) (40:60, v/v) was used as eluent A. Eluent B, consisted of acetonitrile and isopropanol (Burdick & Jackson, USA) both 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 hereafter 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 (ESI) 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^{o}C. APPI was utilized using a krypton discharge lamp (10-eV photons) set with a repeller voltage of +3.5 kV. The dopant utilized for the APPI experiments was acetone (Fisher scientific, Pittsburgh, PA) which was infused at a continuous flow rate of 100µL/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^{o}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 seconds lasting 0.3 seconds. The LockSpray internal reference used for these experiments was Leucine enkephalin (Sigma-Aldrich, St Louis, MO) at a concentration of 5 ng/µL in 50% acetonitrile/ 50% H_2O + 0.1% formic acid (v/v). The reference internal calibrant was introduced into the lock mass sprayer at a constant flow rate of 50µL/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 while 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 it 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 takes place, then more rigorous fragmentation experiments MS/MS CID was 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 (1mg/ml), ranging from 0% excess ^{2}H- labeling up to 2.5% excess ^{2}H-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: H_2O to achieve a final concentration of 0.1 mg/ml. For GC/MS analysis 10 µL 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 x *n*) x 100 where *n* is the number of exchangeable hydrogens, assumed to equal 26, and where 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's manufacturer software (ChemStation & MassLynx respectively). Gene expression data were processed using Ingenuity software (Ingenuity systems, Redwood City, CA) .For the statistical analysis; all the data are presented as ± standard error mean (SEM). Differences between groups were computed by student's *t-test* statistical analysis (GraphPad Prism, La Jolla, CA). Post test analysis for quantifiable variables was conducted using Mann-Whitney *U* non-parametric test with two-tailed *p*-values. Values of $p < 0.05$ was considered as being statistically significant for all the data derived from the experiments herein.

RNA isolation and Real-time quantitative PCR analysis

Liver tissue (~20 mg) was snap frozen in liquid nitrogen and stored at -80 °C. The tissues were homogenized in 600 µL RLT lysis buffer (Qiagen, Valencia, CA) containing 0.1% (v/v) β-mercaptoethanol using a PowerGen 125 homogenizer and 7 x 65 mm 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 µg of RNA using RT^2 First Strand kit (SA Biosciences). Real-time PCR analysis was performed on the 7900HT PCR System (Applied Biosystems, Foster City, CA) with 2x SYBR PCR Master Mix and mouse-specific PCR primers for mouse Scd1, (SABioscienses). Expression levels of stearoyl-CoA desaturase (Scd1) mRNA were normalized to an average of that of mouse beta-actin (Actb), Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), Beta-glucuronidase (Gusb), Hypoxanthine-guanine phosphoribosyltransferase (Hprt1), Peptidylprolyl isomerase A (cyclophilin A) (Ppia) and ribosomal protein 113a (Rp113a) in each sample.

RESULTS

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

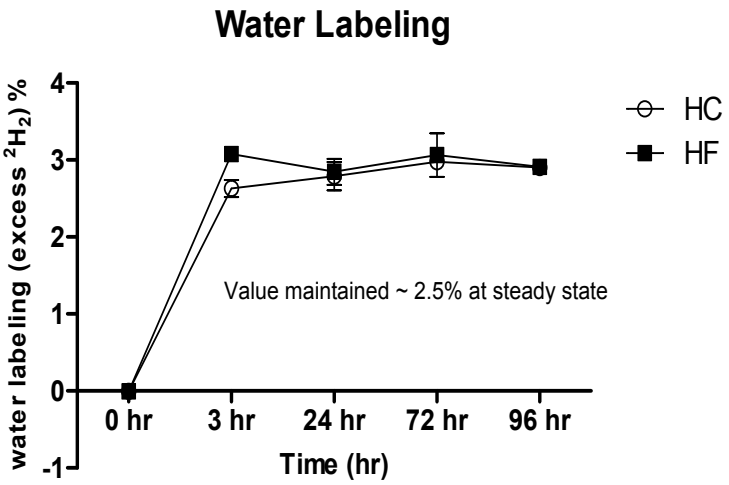
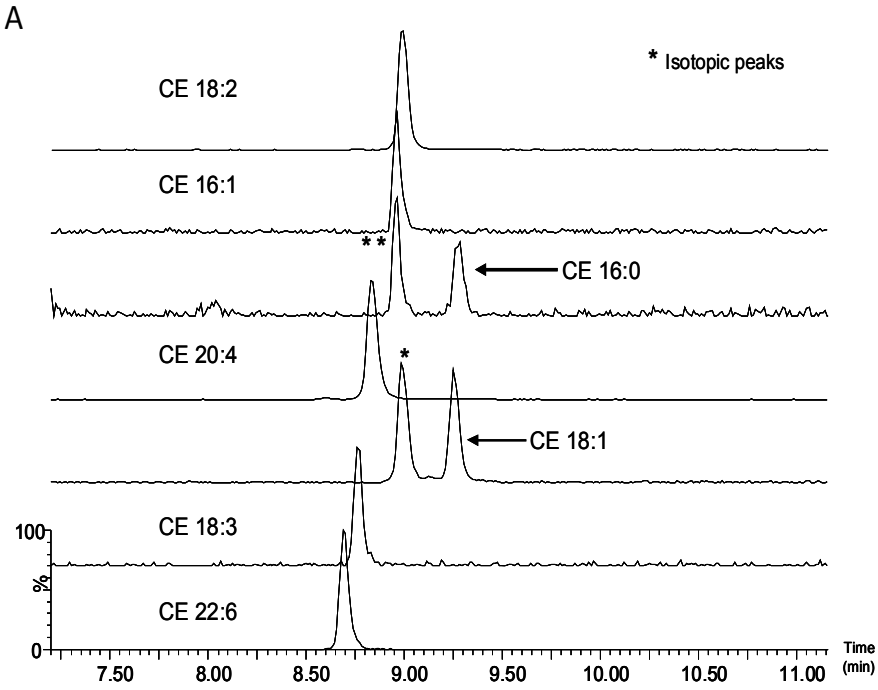


Figure 1. Steady-state labeling of plasma water in mice fed with a HF and HC diets.

Gene expression results from the HC diet revealed an up-regulation in the expression of SREBP1c pathway which contain enzymes involved in *de-novo* lipogenesis, including stearoyl-CoA desaturase (Scd1). In contrast, the SREBP2 pathway was down-regulated. Expression of Scd1 was 69x fold higher ($p < 0.0008$) on the HC diet compared to the HF diet. There is substantial evidence in the literature (45-49) linking over intake of carbohydrates to *de-novo* lipogenesis (DNL). This is accompanied by the synthesis of fatty acids (FA) and the corresponding incorporation into the different phospholipid (PL), triglyceride (TG) and cholesterol ester (CE) pools. When Scd1 is induced, it results in increased desaturation rate of palmitate (FA 16:0) and stearate (FA 18:0) at the 7Z and 9Z position of the fatty acyl chain respectively to give rise to FA 16:1 7Z and FA 18:1 9Z. 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 (FA16:0) containing CE and linoleic acid (FA 18:2 9Z, 12Z) containing CE. This index was increased x 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 (see supplementary figure 3a). Scd1 activity was derived by measuring the desaturation index (DI) between the ratio of palmitoleic acid (FA16:1 7Z)-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 (see supplementary

figure 3b). 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 which can be provided by LC-MS/MS in comparison with GC/MS for the quantifiable flux of both free cholesterol and cholesterol ester 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's but to other lipids such as FFA's, LysoPL's, PL's, DG's, SM's, Cer's and TG's. By the use of this LC/MS methodology the following cholesterol esters in positive ion electrospray mode were detected as ammonium adducts $[M+NH_4]^+$ (Figure 2A and 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, 0.1, 1, 10 and 100μM 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's utilized in this experiment. For the vast majority of cases three orders of isotopic dynamic range was shown (0.1-100 μM) , and only in one instance for CE 18:2 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 since the level of 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 (figure 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 levels of precision are observed at a wide isotopic dynamic range between 3-4 orders as described here. This level of dynamic range was not previously achievable with time of flight mass spectrometers but new developments in the ion detection (analogue to digital converter - ADC) has allowed for the application of this tool for flux analysis. This is a relevant observation as the levels of endogenous cholesterol esters and/or free cholesterol will vary throughout different samples and studies.


A
* Isotopic peaks
CE 18:2
CE 16:1
* *
CE 16:0
CE 20:4
*
CE 18:1
CE 18:3
CE 22:6
100
%
0
7.50
8.00
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9.00
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10.00
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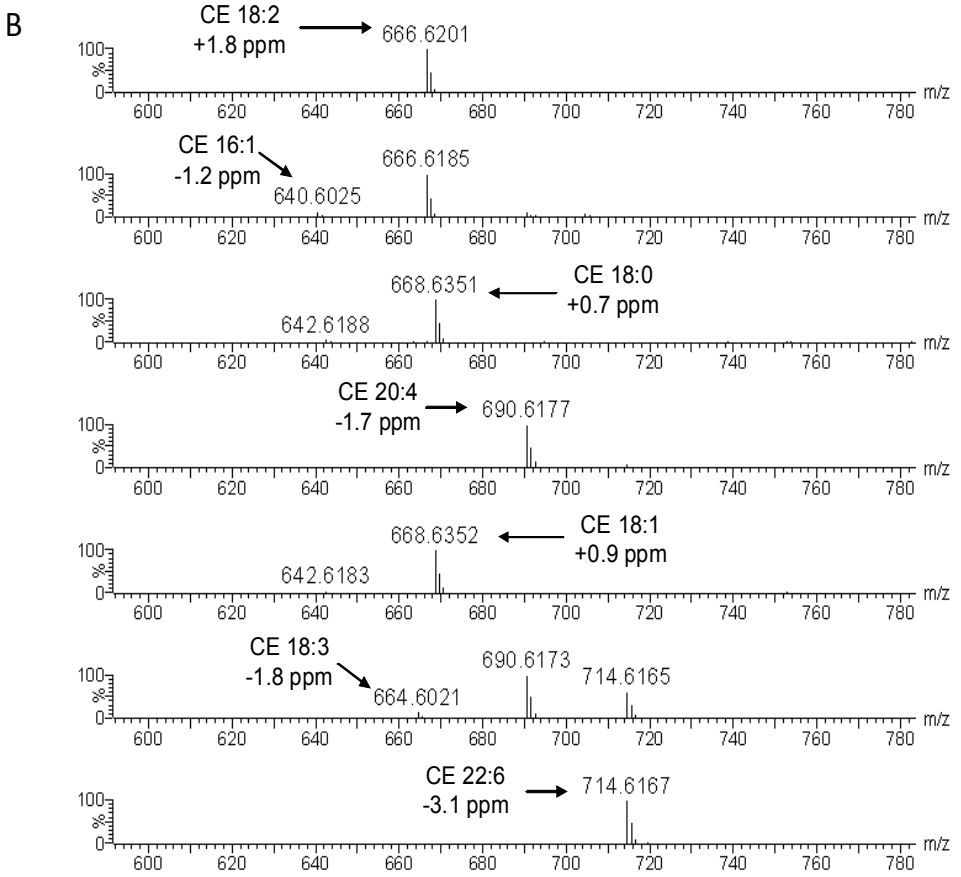


Figure 2: (A) LC/MS profiles of plasma cholesterol esters. Data are shown as the extracted ion chromatogram for each ester, note that the symbols * and ** refer to the M+2 isotopes of cholesterol ester 18:2 and cholesterol ester 16:1, respectively. (B) Full scan MS spectra with accurate mass for the corresponding cholesterol esters.

†*Cholesterol esters detected in electrospray positive ion mode as ammonium adducts*

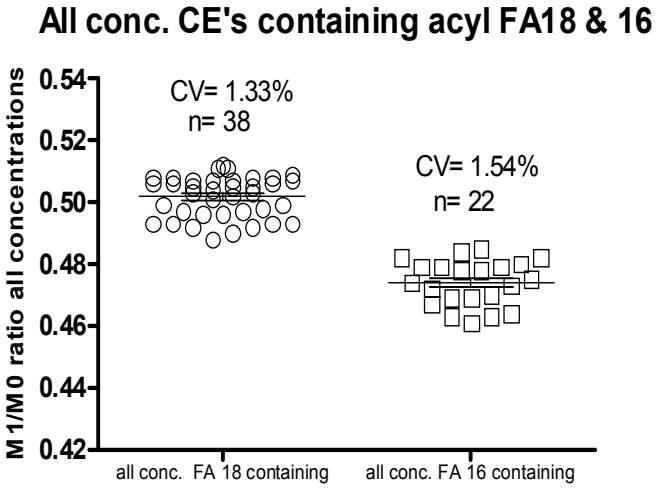


Figure 3: Describes the M1/M0 ratio for all concentrations which contain an acyl FA 18 or 16 for the CE's analyzed in this test. The combination of all concentrations (0.01μM – 100 μM) for the acyl FA 18 containing CE's showed a good coefficient of variation 1.33% (n = 38). With respect to the combination of all concentrations (0.1 μM – 100 μM) for the acyl FA 16 containing CE's showed a good coefficient of variation 1.54% (n=22)

Table1. Isotopic dynamic range measurements for a range of different CE's standards (CE 16:0, CE 16:1, CE 18:0, CE 18:1 & CE 18:2) by LC/ESI-TOF-MS. In all cases a good level of precision was shown. The isotopic dynamic range was between 3-4 orders of magnitude.

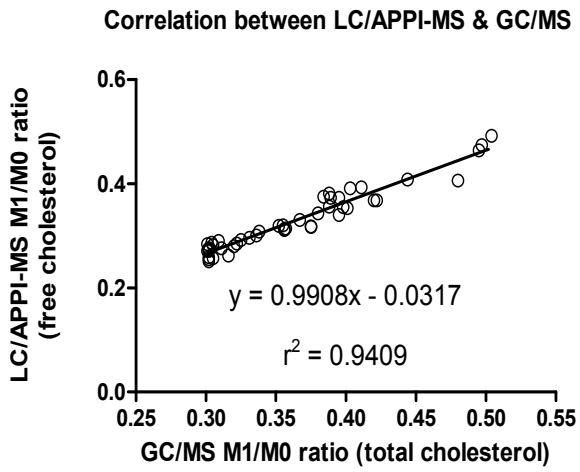
	Cholesterol Ester Concentration (μM)									
	0.01		0.1		1		10		100	
Lipid claass	M1/M0 ratio	cv (%)	M1/M0 ratio	cv (%)	M1/M0 ratio	cv (%)	M1/M0 ratio	cv (%)	M1/M0 ratio	cv (%)
CE 16:0	nd*	nd*	0.488 ± 0.011	2.18	0.469 ± 0.003	0.54	0.479 ± 0.006	1.15	0.467 ± 0.004	0.81
CE 16:1	nd*	nd*	0.475 ± 0.01	2.03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

*cholesterol esters that were not detected at this concentration

Historically GC/MS and GC/IRMS has been the analytical platform of choice for many years and it has 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 –off the isotopic enhancement from a particular ^2H_2O *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 [^2H] cholesterol. Figure 4a describes very clearly the correlation between LC/APPI-MS and GC/MS measurements 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 x + 0.0317. It is important to note the fact 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 latter analytical platform without further sample preparation steps in which the samples do not need to be saponified and free cholesterol can be differentiated from cholesterol esters. Even though we achieved a good level of linearity between the two measurements by the different analytical techniques, Bland-Altman *et al* (50) suggested in their research that a more statistically relevant approach to measure this correlation is by the measurement of the agreement between the two techniques. This 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 figure 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 ^{2}H enhancement can be realized by using this technique but there are certain limitations which need to be noted, if the level of enrichment is below 0.3% then other techniques such as isotope ratio mass spectrometry (IRMS), would be more appropriate. Nonetheless, by the use of IRMS the qualitative information based of the particular lipid class where the label is incorporated and the actual amount is lost as it only measures total incorporation.

A



B

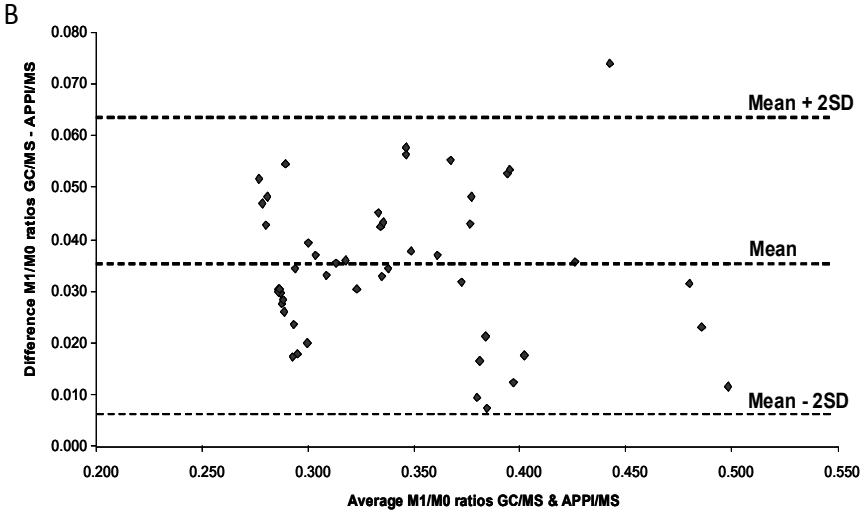


Figure 4. Correlation and isotopic enrichment by ^{2}H between GC/MS (total cholesterol) and LC/APPI-MS (free cholesterol) in the presence of HC/HF diets. (A) Shows the linearity of the isotopic enrichment by GC/MS and LC/APPI-MS for both techniques. (B) Illustrates the differences between GC/MS and 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 +2 SD and Mean -2SD)

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 $^{2}H_2O$ for an *in-vivo* study. The first point of interest was to determine whether it was possible to achieve a level of correlation from the biological samples already described in detail in the experimental section between the two techniques. Therefore, the first step was to analyze the samples for free cholesterol content. It is clear that GC/MS (figure 5A) will only provide information about the total cholesterol labeling in contrast to LC/APPI-MS which is more selective (figure 5B), providing information about the free cholesterol labeling measurement. The important point to note here is the fact that the isotopic enhancement detected by either analytical platform is identical. Therefore, validating the results when compared to the widely adopted analytical techniques such as; GC/MS and GC/IRMS for lower levels of isotopic enrichment. Next, total palmitate was reported by GC/MS (Figure 6A) and when compared to the CE 16:0 LC/MS method (Figure 6B) it showed a very similar trend in both cases, mainly the deuterium label incorporation increased by the HC diet instead of the HF diet. In the case of the measurements made by LC/MS now we have the capability to monitor individual lipid pools rather than observing/measuring incorporation in the total FA pools.

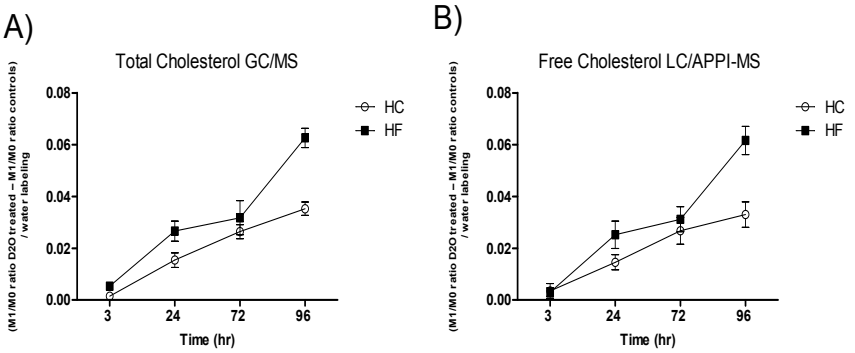


Figure 5. Describes the linearity and correlation of both techniques when used in the context of a biological study. Plot (A) shows the *total* cholesterol ^{2}H incorporation measurement for both diets. Plot (B) shows the *free* cholesterol ^{2}H incorporation measurement for both diets. In both scenarios it shows clearly how there is a higher incorporation of the ^{2}H label in the HF diet vs. the HC diet.

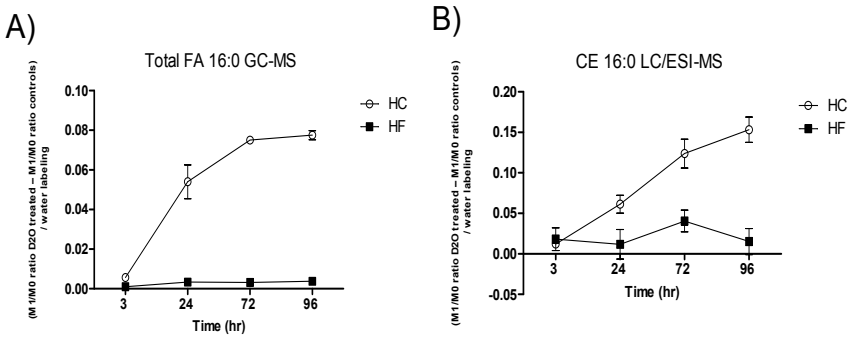
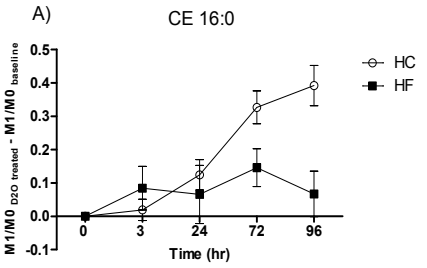
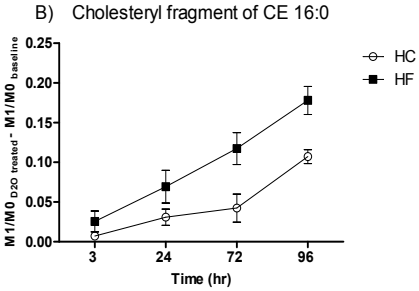
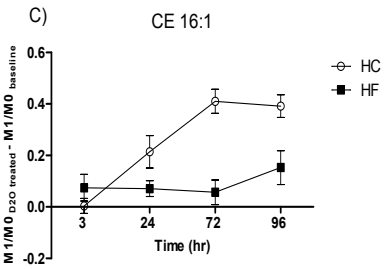


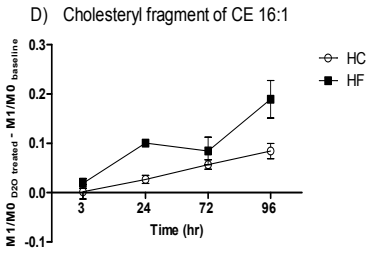
Figure 6. Shows the isotopic labeling correlation in the measurement of total palmitate in plasma by GC/MS vs. the measurement of CE 16:0 in plasma by LC/ESI-MS. Plot (A) provides indication that the palmitate *de-novo* synthesis in the HC diet is very substantial. Plot (B) describes a more selective and complimentary tools in which it is possible to measure the level of incorporation of the labeled palmitate in the CE pool.

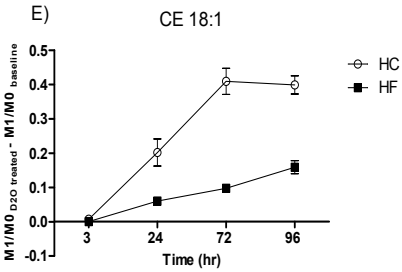
In order to achieve an even higher degree of selectivity, the CE's found by this method were further subjected to CID experiments to obtain the cholesteryl motif fragment ion by electrospray mass spectrometry. This was necessary to determine whether the level of FA synthesis or cholesterol synthesis was responsible for the ^{2}H label incorporation in the CE in question. For both diets the following CE's 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 synthesize cholesterol in the HF diet (0.051 % newly synthesized cholesterol per hour ± 0.003 SD) was higher than in the HC diet (0.031 % newly synthesized cholesterol per hour ± 0.004 SD). In figure 7, it can be divided in several portions. Figure 7A shows how the palmitate is actually driving the incorporation of ^{2}H 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 figure 7C and E, 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 the CE 16:0, CE 16:1 and CE 18:1 are all subjected to CID fragmentation to release the cholesteryl motif (supplementary figure 4). It can be noted that the trend is now reversed (figure 7B, D and F) and the cholesterol in the HF diet is driving the level of incorporation of the ^{2}H 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 has been observed with the saturated and monounsaturated FA's in the cholesterol ester motif. In Figure 7H & J it can be seen that the cholesterol motif is clearly driving the level of incorporation of the ^{2}H 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.

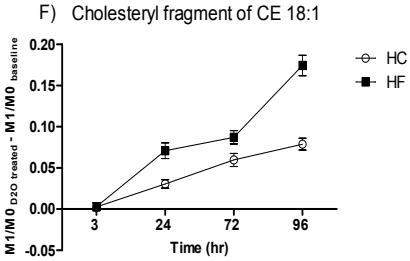

A)
CE 16:0
M1/M0 D2O treated - M1/M0 baseline
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
3
24
72
96
Time (hr)
HC
HF


B) Cholesteryl fragment of CE 16:0
M1/M0 D2O treated - M1/M0 baseline
0.25
0.20
0.15
0.10
0.05
0.00
3
24
72
96
Time (hr)
HC
HF


C)
CE 16:1
M1/M0 D2O treated - M1/M0 baseline
0.6
0.4
0.2
0.0
-0.2
3
24
72
96
Time (hr)
HC
HF


D) Cholesteryl fragment of CE 16:1
M1/M0 D2O treated - M1/M0 baseline
0.3
0.2
0.1
0.0
-0.1
3
24
72
96
Time (hr)
HC
HF


E)
CE 18:1
M1/M0 D2O treated - M1/M0 baseline
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
3
24
72
96
Time (hr)
HC
HF


F) Cholesteryl fragment of CE 18:1
M1/M0 D2O treated - M1/M0 baseline
0.20
0.15
0.10
0.05
0.00
-0.05
3
24
72
96
Time (hr)
HC
HF

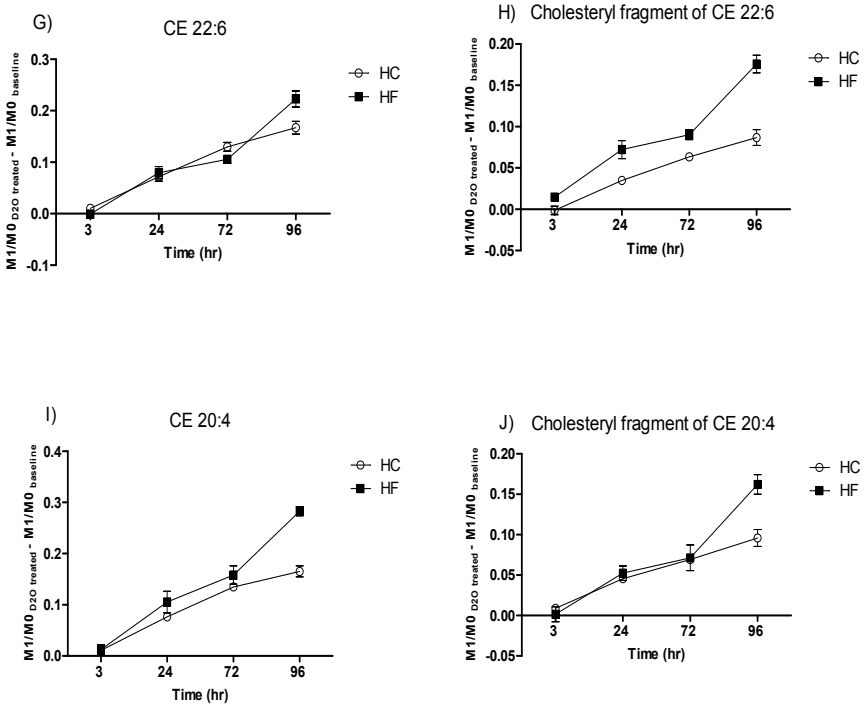


Figure 7. Measurement of ^{2}H isotope labeling for CE's and cholesteryl motif in plasma by LC/ESI-MS/MS for CE16:0, CE 16:1, CE 18:1, CE 22:6 and CE 20:4. Plot (A) by observing the M1/M0 ratios it shows that the palmitate is driving the synthesis in the HC diet, the opposite is true for plot (B) where the cholesteryl motif shows that the ^{2}H incorporation mainly takes place in the HF diet. This event repeats itself for plots (C-F). In plots (G-J), it is significantly different from the rest because now in both cases for the CE 22:6 and CE 20:4 the cholesterol seems to be driving the incorporation of ^{2}H primarily in the HF diet.

Table 2. Represents the percentage of newly synthesized cholesterol measured in plasma by LC/ESI-TOFMS per hour for the two different diets HC/HF.

	% Newly synthesized cholesterol per hour							
Cholesterol Ester[a]	16:0	16:1	18:1	18:2	18:3	20:4	22:6	average ± SD
HC	0.031	0.03	0.027	0.033	0.038	0.029	0.03	0.031 ± 0.004
HF	0.053	0.045	0.052	0.052	0.056	0.05	0.05	0.051 ± 0.003

[a]The corresponding cholesterol esters were fragmented by LC/MS-MS to provide the cholesteryl motif.

DISCUSSION

In summary, a fast and robust method of analysis for quantifying the flux of cholesterol and cholesterol esters *in-vivo* was demonstrated. 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 following 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 will require a much higher level of sample preparation to obtain this detailed degree of information in a much faster manner.

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 preclinical rodent models as higher dosage levels of deuterated water may be utilized per described in this manuscript. In humans and non-human primates the deuterated water administered for flux studies results in a much lower deuterium isotope enrichment ~0.05 atom % excess (25) and therefore it would be more appropriate to utilize IRMS. In our experience this is certainly the case, 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 key aspect for this approach is the fact that the use deuterated water at safe levels does not perturb the metabolic system homeostasis.

A total integrated approach was highlighted in this study, which consisted of detection of genes and enzymes that are up-regulated or down-regulated 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 to this, static measurements of lipids do not provide the level of specificity that can be generated by this flux analysis. For instance, Scd1 which is an enzyme involved in *de-novo* lipogenesis was markedly up-regulated 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 lead 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 approach in terms of lipid quantitative and qualitative composition may be achieved. In turn, the use of this analytical strategy will allow us to simplify the sample analysis and reporting of analytical and biological data in a drug discovery setting. The combination of a platform that comprises gene expression which can guide us towards which biochemical pathway is perturbed by either genetic or novel pharmacological intervention together with a multiplex platform that

enable us to monitor *de-novo* synthesis and have the capability to measure qualitative and quantitative changes proves to be a unique and very desirable platform specially at the very early stages of drug discovery.

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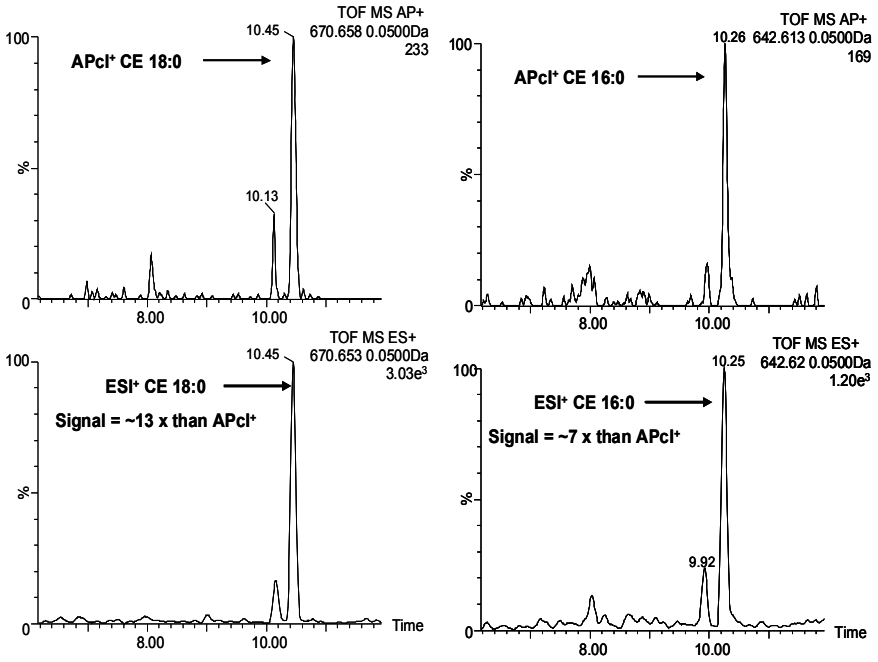
SUPPLEMENTARY INFORMATION

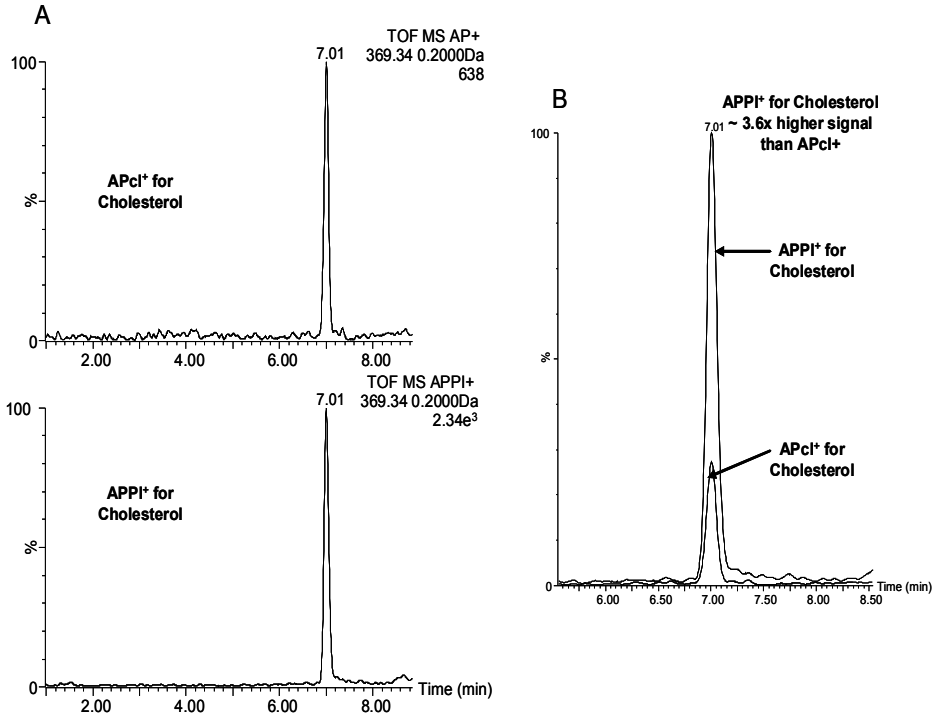
Supplementary Figure 1. Sensitivity comparison between ESI and APcI for equimolar concentrations (1µg/mL) of CE 16:0 and CE 18:0

†Cholesterol esters are detected as $[M+NH_4]^+$

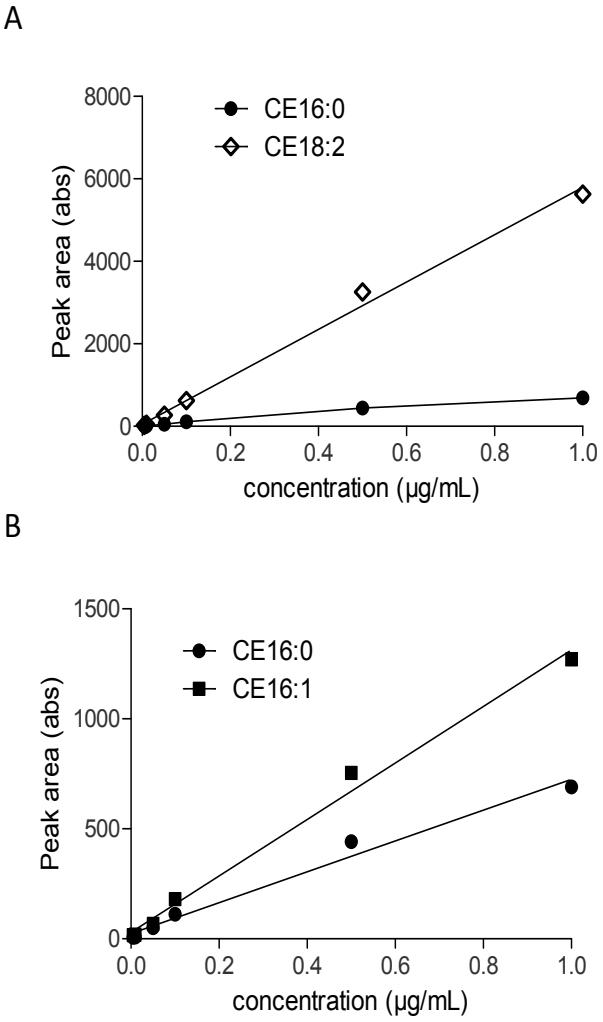


Supplementary Figure 2. (A) Sensitivity comparison between APcI and APPI ionization techniques for cholesterol (standard concentration 1µg/mL). Data was acquired using full scan TOF-MS. (B) Overlay for extracted ion chromatograms ; sensitivity comparison between APcI and APPI ionization with linked axis for cholesterol

††Cholesterol is detected as $[M\text{-}H_2O]^+$



Supplementary Figure 3. (A) Calibration curves for CE 16:0 and CE 18:2 each calibration curve was utilized to calculate the absolute lipogenic index. (B) Calibration curves for CE 16:0 and CE 16:1 each calibration curve was utilized to calculate the absolute desaturation index



Supplementary Figure 4. LC/ESI-MS/MS spectra for CE 16:0, CE 16:1, CE 18:1, CE 18:2, CE 18:3, CE 22:6 and CE 20:4 in C57Bl/6 lipid extracted plasma.