A novel approach to measuring macrophage-specific reverse cholesterol transport in vivo in humans

Marina Cuchel,†,‡ Anna C. Raper,*, Donna M. Conlon,*, Daniel A. Pryma,† Richard H. Freifelder,‡ Rahul Poria,† Debra Cromley,† Xiaoyu Li,*, Richard L. Dunbar,*, Benjamin French,§ Liming Qu,*, William Farver,*, Ching-Chiang Su,‡ Sissel Lund-Katz,*, Amanda Baer,*, Giacomo Ruotolo,† Peter Akerblad,†† Carol S. Ryan,** Lan Xiao,** Todd G. Kirchgessner,** John S. Millar,*, Jeffrey T. Billheimer,* and Daniel J. Rader*

Division of Translational Medicine and Human Genetics, Department of Medicine,§ Department of Radiology,† and Department of Biostatistics and Epidemiology,§ University of Pennsylvania, Philadelphia, PA; Bristol-Myers Squibb R&D,** Princeton, NJ; and AstraZeneca R&D,†† Molndal, Sweden

Abstract  Reverse cholesterol transport (RCT) is thought to be an atheroprotective function of HDL, and macrophage-specific RCT in mice is inversely associated with atherosclerosis. We developed a novel method using 3H-cholesterol nanoparticles to selectively trace macrophage-specific RCT in vivo in humans. Use of 3H-cholesterol nanoparticles was initially tested in mice to assess the distribution of tracer and response to interventions known to increase RCT. Thirty healthy subjects received 3H-cholesterol nanoparticles intravenously, followed by blood and stool sample collection. Tracer counts were assessed in plasma, nonHDL, HDL, and fecal fractions. Data were analyzed by using multicompartimental modeling. Administration of 3H-cholesterol nanoparticles preferentially labeled macrophages of the reticuloendothelial system in mice, and counts were increased in mice treated with a liver X receptor agonist or reconstituted HDL, as compared with controls. In humans, tracer disappeared from plasma rapidly after injection of nanoparticles, followed by reappearance in HDL and nonHDL fractions. Counts present as free cholesterol increased rapidly and linearly in the first 240 min after nadir; counts in cholesterol ester increased steadily over time. Estimates of fractional transfer rates of key RCT steps were obtained. These results support the use of 3H-cholesterol nanoparticles as a feasible approach for the measurement of macrophage RCT in vivo in humans.—Cuchel, M., A. C. Raper, D. M. Conlon, D. A. Pryma, R. H. Freifelder, R. Poria, D. Cromley, X. Li, R. L. Dunbar, B. French, L. Qu, W. Farver, C-C. Su, S. Lund-Katz, A. Baer, G. Ruotolo, P. Akerblad, C. S. Ryan, L. Xiao, T. G. Kirchgessner, J. S. Millar, J. T. Billheimer, and D. J. Rader: A novel approach to measuring macrophage-specific reverse cholesterol transport in vivo in humans. J. Lipid Res 2017, 58: 752–762.

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Although an association between plasma concentrations of HDL cholesterol (HDL-C) levels and CVD has been found in epidemiological studies, the causal basis of this association has been recently questioned by the negative results observed in human genetics studies and clinical trials (1, 2). However, substantial interest remains in the concept that measures of HDL function may be more closely tied mechanistically to atherosclerosis (3). One of the biological functions of HDL is its ability to promote cellular cholesterol efflux and the return of that cholesterol to the liver for excretion in the feces in a process known as reverse cholesterol transport (RCT) (4). By using an in vivo assay in mice that we developed to measure the rate of RCT from macrophages to liver and feces, a large number of studies from multiple laboratories have demonstrated that the rate of in vivo macrophage RCT is a much better predictor of the impact of a genetic or pharmacologic perturbation on atherosclerosis than is the simple measure of HDL-C itself (5). Furthermore, using an ex vivo method developed to assess the cholesterol efflux capacity of HDL from a specific individual, we showed that this was strongly...
inversely associated with prevalent atherosclerosis, even after adjusting for HDL-C levels (6). Similarly, cholesterol efflux capacity of HDL has been shown to be inversely associated with incident CVD (7, 8).

These results suggest that intervention to promote macrophage efflux and RCT might be an effective approach to prevent and treat atherosclerotic CVD. An approach to assess RCT in humans was reported (9), but this method is not macrophage-specific. A method to measure macrophage-specific RCT in humans would be useful, not only in assessing the role of key proteins regulating RCT in humans, but also in evaluating the potential of novel therapeutic approaches targeting cholesterol efflux and RCT.

Nilsson and Zilversmit demonstrated that intravenous (i.v.) injection of rats with a saturated solution of radiolabeled unesterified cholesterol mixed with albumin rapidly disappeared from the blood compartment, primarily due to the uptake by reticuloendothelial (RE) cells (10). Schwartz et al. used a similar preparation to study cholesterol metabolic pathways in humans. Their results were consistent with the rapid clearance of the cholesterol-albumin complexes from the blood compartment and subsequent reappearance of the tracer on circulating HDL as free cholesterol (FC), suggesting that this approach may specifically measure the efflux of cholesterol from macrophage cells to HDL as sole acceptor (11–14).

Based on these data, we hypothesized that cholesterol nanoparticles made of albumin-bound H-cholesterol could be used to target H-cholesterol selectively to macrophages in vivo and permit the assessment of macrophage-specific cholesterol efflux in vivo in humans. We present here the results of preclinical validation studies and a feasibility study in humans that were conducted to test the validity and feasibility of such approach.

METHODS

Preclinical studies

Animal studies were generally conducted in C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) fed a standard chow diet (catalog no. 5020, Research Diets, New Brunswick, NJ) ad libitum, unless otherwise stated. Cholesterol nanoparticles were prepared similarly to that for human studies (see below), unless otherwise stated, and injected via tail vein (100 µl solution containing 30 µCi/ml H-cholesterol). Blood and/or tissue were obtained at several time points postinjection. Plasma radioactivity was counted directly, and tissue radioactivity was determined after extraction by using SOLVABLE (PerkinElmer, Waltham, MA) according to the manufacturer’s directions. Reconstituted HDL was prepared as previously described (15) and administered intraperitoneally at a dose of 3 mg apoA-I/200 µl PBS to age- and gender-matched mice.

Validation studies using the liver X receptor (LXR) agonist BMS-779788 were conducted at Bristol-Myers Squibb (Princeton, NJ). Male C57Bl/6J (Jackson Laboratory) and LXR α (−/−), LXR β (−/−), and LXR α/β double (−/−) (Bristol-Myers Squibb) mice on C57Bl/6 background were single-housed for acclimation and throughout the compound administration phase of the study. Water and standard rodent chow (Harlan Teklad, Frederick, MD) were provided ad libitum. Age-matched mice (12–24 weeks of age; n = 2 or 3 per group) were randomly assigned into the following treatment groups: vehicle [0.5% carboxymethyl cellulose and 0.2% Tween 80 (Sigma-Aldrich, St. Louis, MO)], or 30 mg/kg/d BMS-779788 and dosed at 9 AM, once a day, for 7 days by oral gavage. At 5 h after final dose, animals were anesthetized with inhaled 4% isoflurane and 0.06 ml of BSA (Sigma-Aldrich): 3H-cholesterol nanoparticles (60 µl per animal) were injected intravenously via the orbital plexus of 30 g nonfasted animals. Cholesterol nanoparticles were prepared by mechanical stirring of 972 µl 3% BSA in 0.9% saline with drop-wise addition of 108 µl (1.08 mg/ml, 2 Ci/mmol) of [3H]cholesterol in ethanol (PerkinElmer) at 600 rpm for 15 min at room temperature. Anesthesia concentration was reduced to 1% after injection and maintained throughout the study period in a humidified anesthesia chamber with tail access ports. Blood samples (25 µl) were collected into microvette plasma isolation tubes (Sarstedt, Nümbrecht, Germany) by venous tail nick from each mouse at 5, 15, 30, 45, 60, 70, 90, 105, 120, 135, and 150 min postinjection. After isolation by centrifugation at room temperature, 10 µl plasma was added to 5.0 ml Ecolite scintillation fluid (MP Biomedical, Santa Ana, CA), and radioactivity was determined by liquid scintillation counting.

Kupffer cells (KCs) were isolated from mouse liver as previously described (16). Briefly, mice were anesthetized, and the inferior vena cava was cannulated. Subsequently, the vena portae was ligated, and the liver was perfused for 5 min with Liver Perfusion Buffer (Invitrogen, Tokyo, Japan) until the liver turned pale, then digested in situ with Liver Digest Solution (Invitrogen) for approximately 10 min. Parenchymal cells were isolated after mincing the liver in Hepatocyte Wash Medium (Invitrogen), filtering through nylon gauze, and centrifuging for three times 5 min at 50 g. The pellets consisted of pure (≥99%) parenchymal cells as judged by phase-contrast light microscopy. The supernatants were centrifuged for 10 min at 500 g in order to harvest the nonparenchymal cells. By means of centrifugal elutriation, the endothelial cells (ECs) and KCs were separated according to the method of Nagelkerke et al. (16).

KCs were depleted in mice by using clodronate as previously described (17). Briefly, mice were i.v. injected with 200 µl of liposome-encapsulated clodronate (1 mg), which is known to deplete KCs, or PBS encapsulated in multilamellar liposomes (ClodronateLiposomes.com, Amsterdam, The Netherlands). KC depletion was assessed by using quantitative RT-PCR to quantify liver F4/80 and Nrampl mRNA. Results were normalized by mRNA levels of β-actin. After 48 h, the mice were injected via tail vein with 100 µl of 3H cholesterol nanoparticles and were bled and euthanized either 1 or 10 min after injection. Radioactivity was measured in plasma, liver, and red blood cells as described above.

The cholesterol efflux of isolated KCs from C57BL/6 mice was assessed according to an established protocol using chisterionic acid and 22-hydroxycholesterol to upregulate the ABCA1 transporter and either apoA-I or the HDL fraction obtained by precipitation of ApoB using polyethylene glycol (PEG), as an acceptor (18).

The University of Pennsylvania or the Bristol-Myers Squibb Institutional Animal Care and Use Committee approved the animal experiments, and all experiments were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Cholesterol nanoparticles preparation for human studies

Albumin-bound H-cholesterol (cholesterol nanoparticles) was prepared in the Cyclotron Facility of the University of Pennsylvania under strict operating procedures that aimed to create complexes with uniform size characteristics. GMP cholesterol was obtained from Avanti Polar Lipids (Alabaster, AL), and H-cholesterol (40–60 Ci/mmol) was from Perkin Elmer. Cholesterol nanoparticles were provided ad libitum. Age-matched mice (12–24 weeks of age; n = 2 or 3 per group) were randomly assigned into the following treatment groups: vehicle [0.5% carboxymethyl cellulose and 0.2% Tween 80 (Sigma-Aldrich, St. Louis, MO)], or 30 mg/kg/d BMS-779788 and dosed at 9 AM, once a day, for 7 days by oral gavage. At 5 h after final dose, animals were anesthetized with inhaled 4% isoflurane and 0.06 ml of BSA (Sigma-Aldrich): 3H-cholesterol nanoparticles (60 µl per animal) were injected intravenously via the orbital plexus of 30 g nonfasted animals. Cholesterol nanoparticles were prepared by mechanical stirring of 972 µl 3% BSA in 0.9% saline with drop-wise addition of 108 µl (1.08 mg/ml, 2 Ci/mmol) of [3H]cholesterol in ethanol (PerkinElmer) at 600 rpm for 15 min at room temperature. Anesthesia concentration was reduced to 1% after injection and maintained throughout the study period in a humidified anesthesia chamber with tail access ports. Blood samples (25 µl) were collected into microvette plasma isolation tubes (Sarstedt, Nümbrecht, Germany) by venous tail nick from each mouse at 5, 15, 30, 45, 60, 70, 90, 105, 120, 135, and 150 min postinjection. After isolation by centrifugation at room temperature, 10 µl plasma was added to 5.0 ml Ecolite scintillation fluid (MP Biomedical, Santa Ana, CA), and radioactivity was determined by liquid scintillation counting.

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were prepared by adding 15 ml of 1% human serum albumin to a solution of cholesterol in a vial (150 nl unlabeled cholesterol, 1 mg/ml in ethanol, and 75–200 μl 3H-cholesterol, 1 nCi/ml). The solution was stirred for 15 min at 60 rpm and then filtered through a 0.22 μm filter to obtain the final product. The preparation, containing approximately 50–75 μCi 3H-cholesterol and 150 μg FC, was administered i.v. as a bolus within 1 h of being prepared. To determine the size of the cholesterol nanoparticles, analyses were performed on an unlabeled solution by using a laser-diffraction particle size distribution analyzer (catalog no. LA-950A, software version 5.00, Horiba, Kyoto, Japan) and via dynamic light-scattering analysis (Wyatt DynaPro plate reader, Wyatt Technology, Santa Barbara, CA; software Dynamics version 6.12.0.3 for data collection and evaluation). The two methods gave comparable results. A mean hydrodynamic radii (Rd) of the cholesterol nanoparticles was determined to be 80–100 nm.

Clinical protocol

We enrolled men and women ages 18–70 years (n = 30) in good overall health and with a range of HDL-C levels. Female subjects were included only if of nonchildbearing potential. Use of lipid-lowering drugs within the 6 weeks before dosing or during the study and/or prescription or nonprescription drugs within 2 weeks before dosing was exclusionary.

Eligible subjects were admitted to the clinical research center within 30 days of screening. Subjects were asked to fast for a minimum of 12 h before their research unit admission and remain in the research unit for a total of 27 h. Upon admission, cannulation of the antecubital vein was performed in both arms (one was used for radiolabeled cholesterol administration and the other for blood sampling). Subjects were provided with a nonfat breakfast 3 h before the start of the tracer administration and a low-fat lunch and dinner at scheduled times after the injection of the tracer. Cholesterol nanoparticles were prepared as described above and administered within 1 h of preparation as a rapid i.v. bolus. Blood samples (18 ml) were collected in EDTA-containing vacutainer tubes before and several times after tracer administration and immediately placed on ice. Subjects were discharged from the research unit after the 24 h blood draw and returned to the research unit at 48 h, 72 h, 96 h, and 8 days after dosing for blood sampling and stool collection. The study was conducted under Food and Drug Administration Investigational New Drug Application 115,938 and approved by the University of Pennsylvania Institutional Review Board, and all subjects signed the approved informed consent.

Sample processing

Blood samples were placed in ice and centrifuged within 20 min. A 2 ml aliquot of plasma was added in a 15 ml glass extraction tube containing 2 ml of HDL-precipitating solution which contains phosphotungstic acid (Thermo Scientific, Middletown, VA), vortexed, and centrifuged. The supernatant (HDL fraction) was measured as previously described (6). Lipoproteins were extracted by using standard Bligh-Dyer methodology (19). Plasma, HDL, and nonHDL lipid extracts were then further processed to separate FC and cholesterol ester (CE) fraction with silica acid column chromatography. Red blood cells were washed with saline and reisolated, and lipids were extracted as described above. Stool collections were weighed and homogenized. Samples of >400 g from 25 individuals were homogenized, and a 3 g aliquot was saponified, lipids were extracted with hexane, and the cholesterol fraction was obtained by silica acid column chromatography. The presence of bile pigment prevented the determination of tracer in the stool bile acid fraction. Radioactivity in cholesterol extracts was determined by using liquid scintillation counting. Lipid and lipoproteins were measured by using standard commercial kits. Ex vivo cholesterol efflux capacity of the HDL fraction was measured as previously described (6).

Multicompartmental analysis

The multicompartmental analysis was conducted by using the WinSAAM modeling program. The model (Fig. 1), based on the cholesterol model developed by Schwartz et al. (14), features efflux of FC to HDL from cells of the RE system as a key measure of the RCT process. Because our protocol used a single tracer and did not involve bile sampling, the Schwartz model was modified so that all parameters could be identified with confidence. Previously published results provided estimates of the initial parameters utilized during the fitting process (13, 15). FC on HDL can transfer to nonHDL particles, red blood cells, or peripheral tissue cholesterol (a composite of cholesterol from all nonliver tissues, liver. The numbers within each circle, consistent with those used previously (15), are used to identify each compartment in the WinSAAM program and have no other relevance. The asterisk represents the site of injection of the cholesterol tracer. U(12) represents input of newly synthesized cholesterol into the system via liver. Arrows represent cholesterol transport pathways between compartments. Arrows leading away from compartments that do not connect to another compartment represent irreversible transfer out of the system. In the case of compartment 25, this may represent conversion of FC to storage as cholesteryl ester. In the case of compartment 12, this may indicate excretion from liver in bile. nHDL, nonHDL; RBC, red blood cell; RES, RE system.

![Figure 1: Multicompartmental model used to determine rates of cholesterol transfer between different cholesterol compartments.](image-url)
including steroidogenic tissues) or be esterified, at which point it either transfers to non-HDL particles or is taken up by liver. Cholesterol taken up by liver is either resecreted into plasma as FC onto HDL or non-HDL, accounting for recycling of the labeled cholesterol tracer or excreted in feces.

Statistical analysis

The statistical analysis of data obtained in the human study was performed with R (Version 3.0.1; R Development Core Team, Vienna, Austria). Subject characteristics were summarized by using standard descriptive statistics. The slope of appearance between 60 and 240 min postinjection was estimated for each subject by using a linear model for relevant parameters. Pearson coefficient was used to assess correlation between variables of interest. Statistical significance was defined as $P < 0.05$.

RESULTS

Preclinical studies

After the bolus administration of nanoparticle cholesterol in WT mice, the tracer rapidly disappeared (half-life < 1 min) from circulation, reaching a nadir within 10 min postinjection, followed by reappearance in plasma that plateaued by 2 h and then began declining at 6 h postinjection (Fig. 2A). Ten minutes after injection, the cholesterol tracer was distributed primarily in the liver and the spleen (Fig. 2B). By 6 and 24 h after injection, the total counts in the liver and spleen were decreased, and counts in other tissues, such as adrenal, kidney, and heart, were detectable (Fig. 2B). Isolation of liver parenchymal and nonparenchymal cells showed that, on a milligram of protein basis, the amount of tracer in nonparenchymal cells was >20 times that of parenchymal cells (8.7 ± 3.8 vs. 0.4 ± 0.1%/mg protein) (Fig. 3A). Six h after injection, the tracer amount present in the nonparenchymal cells had decreased to 1.8 ± 0.5%/mg protein (Fig. 3A), whereas that in parenchymal cells remained essentially unchanged (0.4 ± 0.08% injected dose/mg).

We assumed that the liver nonparenchymal cells responsible for uptake of the cholesterol nanoparticles were cells of the RE system, namely KCs. To further substantiate our assumption, KCs were depleted from mice by using clodronate liposomes. After 48 h of clodronate treatment, KCs were depleted by >90%, as judged by decrease in mRNA of macrophage-specific markers F4/80 and Nramp1 (data not shown). The removal of cholesterol nanoparticle from the plasma of mice that received PBS is extremely rapid with a half-life of <1 min and with <3% of plasma counts remaining after 10 min (Fig. 3B). The depletion of KCs in mice that received clodronate resulted in a reduced clearance of the tracer, with 21% of plasma counts remaining after 10 min (Fig. 3B). This was accompanied by a 2- to 3-fold increase in tracer in the red blood cell fraction (Fig. 3C) at both 1 min (8.1 ± 0.9% vs. 4.5 ± 0.8%) and 10 min (4.5 ± 1.5% vs. 1.3 ± 0.2%). Importantly, the uptake by the liver at 1 min was reduced by 50% (40 ± 2% vs. 19 ± 5% of injected dose) and remained decreased at 10 min (54 ± 2% vs. 30 ± 3%) (Fig. 3D).

To test their ability to efflux cholesterol to known acceptors, isolated murine KCs demonstrated physiological cholesterol efflux to HDL and apoA-I that was responsive to LXR activation with 9-cis-retinoic acid/22-R hydroxysterol to stimulate ABCA1 expression comparable with that seen in macrophages (Fig. 4).

To validate the method, we conducted studies using two interventions expected to increase cholesterol efflux and RCT. The effect of LXR agonism, a mechanism known to stimulate RCT in other assays, was evaluated first. C57Bl/6 mice were treated with the LXR agonist BMS-779788 (20) or vehicle for 7 days, followed by the administration of $^3$H] cholesterol nanoparticles. Within 30 min, 98% of the injected label was cleared from the plasma in both treatment groups. Label subsequently reappeared in the vehicle-treated group, and this rate was increased in BMS-779788-treated animals (Fig. 5A). Similar results were obtained in LXRβ KO animals (Fig. 5C). However, no reappearance of label in plasma was apparent in either vehicle or LXR

![Fig. 2.](image_url)
agonist-treated groups in LXRα KO and LXRα/β double-KO mice (Fig. 5B, D). This suggests that in this model, both basal and LXR agonist stimulated macrophage cholesterol efflux is LXRα-dependent, but not LXRβ-dependent.

Second, mice were infused with apoA-I/phospholipid complexes (rHDL) or saline as we previously described (15). As expected, rHDL infusion resulted in a significant increase in cholesterol mass in plasma (Fig. 6A). Notably, rHDL infusion also resulted in a significant increase in the rate of reappearance of cholesterol tracer after nanoparticle injection, with plasma counts at 2 h 3-fold higher in the rHDL group compared with the saline control group (Fig. 6B).

**Clinical studies**

A total of 30 healthy subjects were enrolled into the study. Demographic characteristics and baseline lipid profiles are shown in Table 1.

After the i.v. administration of particulate cholesterol, the tracer counts in plasma reached a nadir approximately 1 h postinjection and appeared at a linear rate.
over approximately the next 3 h, after which they plateaued and then decreased steadily over time (Fig. 7A). The percentage of counts present in plasma as FC (the form in which the tracer is injected) rapidly declined, whereas the tracer present as CE increased steadily, until reaching an equilibrium, at which it represented approximately 70% of the total tracer amount in circulation (Fig. 7B). Similar results were observed in HDL and nonHDL fractions.

The slope of initial reappearance (between 1 and 4 h postinjection) was steeper and the specific activity was higher for counts present as FC than CE (Table 2 and Fig. 8). Counts were initially higher in the HDL fraction than in the nonHDL fraction, suggesting that the tracer first appeared in HDL.

Twenty-five subjects volunteered to collect their stools over a 5-day period postinjection. The counts measured in the neutral sterol fraction were approximately 50 times background and contained 1.95 ± 0.92% of the injected dose.

Mass and tracer data from plasma, HDL, and nonHDL fractions, as well as from blood cells and neutral fecal sterol, were used to determine fractional transfer and transport rates using multicompartmental modeling. Fractional transfer rates for key steps of the RCT, namely the efflux of FC from macrophage to HDL, the esterification of HDL-FC to HDL-CE, and the transfer of CE from HDL to nonHDL, were calculated and are shown in Table 3. Based on the modeling, FC taken up by the macrophage on labeled nanoparticles was transferred (effluxed) to HDL relatively
slowly at an estimated rate of 0.028 pools/hour (approximately 3% of the FC in the RE cells was transferred into HDL per hour); the esterification of HDL-FC to HDL-CE occurred at 0.249 pools/h, and the transfer of HDL-CE to nonHDL occurred relatively slowly, at 0.171 pools/h (approximately 17% per hour). In contrast, and as expected, the transfer of FC from HDL to nonHDL very rapidly occurred at 8.044 pools/hour (approximately 800% per hour). In absolute terms, the transfer of FC from HDL to nonHDL was high, at 9,050 μmol/h. In contrast, HDL-FC conversion to HDL-CE was relatively small, being 271 μmol/h. The transfer of CE from HDL to nonHDL, which was quantitatively greater at 568 μmol/h, accounts for the transfer of CE derived from esterification of HDL-FC and CE back transferred from nonHDL.

DISCUSSION

We report here a method to trace macrophage efflux and RCT in humans in vivo. Our preclinical studies demonstrated that, after injection, radiolabeled cholesterol tracer administered as cholesterol nanoparticles rapidly disappeared from circulation and was taken up primarily by cells of the RE system, with efflux back into the circulation over time. Furthermore, we demonstrated that KCs were able to efflux cholesterol with pathways that are similar to those observed in a commonly used macrophage cell line (6, 18) and can be a suitable surrogate for macrophages.

Depletion of KCs by clodronate reduced the radiolabeled cholesterol nanoparticles in the liver by >50% at 1 min. This was less than the expected reduction based on the 70–80% uptake estimated by Zilversmit (11), which was probably due to at least three reasons. First, some liver uptake may have been due to residual KCs. Second, the increase in tracer observed in the red blood cell pellet of mice injected with clodronate suggests that a portion of the FC present in the nanoparticles not immediately removed may have been dissolved in the plasma, interacted with red blood cells, and exchanged with lipoproteins, with resultant receptor-mediated uptake by the liver. Finally, it is likely that in the absence of KCs, some of the cholesterol nanoparticles may have been taken up by liver sinusoidal ECs. Consistent with this possibility, Park et al. have recently demonstrated that poly(lactic-co-glycolic acid) nanoparticles, similar in size to the cholesterol nanoparticles used in our studies, are taken up primarily by KCs, but upon

| TABLE 1. Demographic characteristics and lipid profile of study subjects (n = 30) |
|-----------------------------------------------|
| Age, years                                     | 36.1 ± 14.5 |
| Male, n (%)                                    | 28 (95)    |
| Race                                          |            |
| Caucasian, n (%)                              | 19 (63)    |
| Black, n (%)                                  | 9 (30)     |
| Other, n (%)                                  | 2 (7)      |
| BMI (kg/m²)                                   | 25.6 ± 4.1 |
| Systolic BP (mmHg)                            | 120 ± 10   |
| Diastolic BP (mmHg)                           | 73 ± 9     |
| Lipid profile (mg/dl)                         |            |
| Total Cholesterol                             | 173 ± 42.9 |
| LDL-Cholesterol                               | 105 ± 33   |
| HDL-Cholesterol                               | 50 ± 16    |
| Triglycerides                                 | 70 (56–110) |
| Apo A-I                                       | 128 ± 28   |
| Apo B                                        | 76 ± 23    |
| Lp(a)                                         | 21 (9–54)  |
| Cholesterol efflux                            | 0.608 ± 0.1556 |

Data are expressed as mean ± SD or median (interquartile range), if not otherwise specified. Cholesterol efflux: ex vivo cholesterol efflux capacity is reported as value normalized to the value of a pooled control serum, as previously described (8). BP, blood pressure.
depletion of these cells, there is an 8-fold increase in the uptake by sinusoidal ECs (21).

The rapid reappearance of the tracer suggests that part of the radiolabeled cholesterol taken up by the RE system cells does not get extensively esterified and stored, and it is probably compartmentalized at the membrane level and rapidly available to be effluxed to acceptor particles. Importantly, there is no apparent direct exchange of tracer between the RE cells and the parenchymal cells, as supported by the finding that the amount of tracer in the liver nonparenchymal cells decreased over the 6 h of the study, but remained unchanged in the parenchymal cells, suggesting that the tracer was released back into circulation and equilibrated with body cholesterol pools.

Pieters et al. elegantly investigated macrophage-specific RCT in rats using \(^{3}\text{H}\) cholesteryl oleate-labeled oxidized LDL. After i.v. administration in rats, these particles were taken up preferentially by the KCs, their CE was hydrolyzed, and FC reappeared in circulation, to be eventually secreted in the bile (22). We did consider the use of modified LDL particles; however, the GMP preparation of such particles for human use is not straightforward. Our choice to use cholesterol nanoparticles for the assessment of the RCT pathway was driven by the feasibility of their GMP preparation for human use and the existence of supporting data not only from previous animal studies by Nilsson and Zilversmit (10), but also critical from human proof-of-concept data generated by Schwartz et al. (11–14). Importantly, our data support the concept that the FC bound to albumin in these nanoparticles was taken up before extensively exchanging with red blood cells.

To validate the use of cholesterol nanoparticles as a method to assess RCT in vivo, we used two interventions that are known to promote RCT: LXR agonists (20, 23) and rHDL (24, 25). Administration of cholesterol nanoparticles in mice that received rHDL was associated with an increase in plasma counts as compared with animals that received saline. Similar results were observed in mice treated with an LXR agonist as compared with animals that received vehicle. Interestingly, an increase in radioactivity was not observed in LXR\(\alpha\) KO and LXR\(\alpha\)/\(\beta\) double-KO mice, suggesting that the initial movement of cholesterol out of macrophages is primarily driven by LXR\(\alpha\) in this model. This is somewhat unexpected because both LXR isoforms have been shown to stimulate cholesterol efflux in response to LXR agonists in peritoneal macrophages in vitro (26, 27). Moreover, the absence of basal cholesterol efflux in mice lacking LXR\(\alpha\) must be interpreted in light of the existence of both ABCA1- and ABCG1-dependent and-independent efflux mechanisms, including SR-B1-mediated and aqueous diffusion. However, in vitro studies indicate that, in contrast to normal macrophages, ABCA1 and ABGG1 are the predominant efflux pathways in cholesterol-loaded cells, with SR-B1 and aqueous diffusion playing more minor roles (28).

![Fig. 7](image-url)

**Fig. 7.** A: Observed \(^{3}\text{H}\)-cholesterol expressed as cpm/\(\mu\)mol in human plasma, nonHDL, and HDL fractions over time (hours). B: Percentage of \(^{3}\text{H}\)-cholesterol circulating as FC and as CE in plasma over time. Values are reported as mean ± SD (represented by gray shaded area) (\(n = 30\)).

| TABLE 2. Slope of reappearance: increase in tracer-specific activity (cpm/\(\mu\)mol) in nonHDL and HDL fractions for 1 h increase in time (60–240 min) |
|---------------------------------------------------------------|
| Non-HDL | HDL |
| Total counts | 34.0 (26.0, 52.9) | 62.1 (40.2, 85.5) |
| Counts as FC | 119.4 (97.0, 196.4) | 121.9 (71.4, 184.6) |
| Counts as CE | 5.6 (4.3, 8.3) | 32.1 (23.3, 60.0) |

Data are expressed as median (Q1–Q3).
nanoparticles in vivo may also efflux cholesterol primarily through ABCA1 and ABCG1, both of which are regulated by LXR. Nevertheless, both the LXR agonist and rHDL studies support the concept that this method is able to assess changes in RCT. It is also noteworthy that a dose-dependent increase in macrophage RCT by an LXR agonist was detectable by using this method and that it mirrored the dose-response for inhibition of atherosclerosis in mouse models (29).

Data obtained from the study in healthy volunteers are consistent with those reported by Schwartz et al. (11–14). After the tracer nadir, approximately 1 h after bolus administration, the specific activity of the ³H-cholesterol (as total, FC, and CE counts) was initially higher in the HDL fraction than in any other fraction, suggesting that tracer reappearance in circulation is initially mediated by the HDL fraction. These data are consistent with a model in which the tracer is effluxed back in circulation into HDL particles, where it is esterified and/or exchanged with apoB-containing lipoproteins (14).

Reappearance of the tracer showed a good linearity up to 4 h postinjection, that was then lost. The observed

![Graph](image-url)

**Fig. 8.** Observed ³H-cholesterol expressed as cpm/µmol in human plasma, nonHDL, and HDL fractions over the first 24 h. A: Total counts. B: Counts present as FC. C: Counts present as CE. Values are reported as mean (n = 30).

| Fractional transfer (pools/h) | HDL-FC to nonHDL-FC | HDL-CE to nonHDL-CE |
|------------------------------|----------------------|----------------------|
| 0.028 ± 0.024                | 8.044 ± 3.728        | 0.249 ± 0.075        |
| Absolute transfer (µmol/h)   | n.d.                 | 9.050 ± 4.660        |
| Pool size of originating compartment (µmol) | n.d.             | 1,145 ± 375          |

Data are mean ±SD (n = 30). n.d., not determined. The transfer rates listed correspond to the following transfer rates in the model shown in Fig. 1: Macrophage-FC to HDL-FC (compartment 25 to 4); HDL-FC to nonHDL FC (compartment 4 to 5); HDL-FC to HDL-CE (compartment 4 to 9); HDL CE to nonHDL-CE (compartment 9 to 7).
variability in the slope of reappearance may depend on both the ability of the cell to efflux FC, as shown in mice by the increase in the slope after the administration of the LXR agonist, as well as the availability of the acceptor (i.e., HDL), as shown by the increase in the plasma tracer amount after the administration of rHDL.

The loss of linearity of the rate of reappearance was likely due to the rapid esterification of FC into CE by LCAT and the exchange with other lipoproteins. The presence of the tracer as CE in HDL increased rapidly and linearly during the initial phase of reappearance. This parameter may represent an integrated readout of the ability to efflux FC into HDL and esterification to CE. Of note, the CE slope in the HDL fraction was steeper and the specific activity was higher in the HDL fraction as compared with the nonHDL fraction.

Multicompartmental modeling can provide an integrated overview of the entire RCT process and allow an appropriate interpretation of the results obtained. The model used is consistent with the cholesterol model developed by Schwartz et al. (14), and the results are consistent with what has been reported previously (13, 14). Of interest is the fact that, through multicompartmental modeling, we can obtain an in vivo accurate estimate of the three key steps of RCT that affect the initial reappearance of the tracer in circulation, namely, the fractional transfer rate of the tracer as FC from the RE system compartment to HDL (that depends on the ability of the macrophage to efflux cholesterol and the availability of the acceptor), the fractional transfer rate of the tracer present in HDL from FC to CE (that is LCAT-dependent), and the fractional transfer rate of the HDL-CE into nonHDL particles (that reflects cholesteryl ester transfer protein activity). The possibility of estimating these critical RCT steps will be an invaluable tool for the assessment of novel therapeutic approaches aimed to affect RCT.

We demonstrated that, after the administration of cholesterol nanoparticles, radiolabeled tracer is excreted and can be measured in stool. Although counts in neutral fecal sterols were markedly elevated over the preinjection background counts, we observed a marked interindividual variability. Subject compliance for stool collection as well as normal variation in bowel movement may have played a role and will need to be taken into consideration when designing future studies.

In conclusion, the preclinical data validate the use of cholesterol nanoparticles as a method to assess RCT in vivo animal models and offer a solid rationale for the use of this approach in humans. The results of the human study support the feasibility and safety of this innovative approach and suggest that this method may be used to measure the rate of macrophage RCT in vivo in humans.

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