Materials and Methods

Materials: [Methyl-^{14}C] choline chloride, [1, 2-^{3}H (N)] free cholesterol (FC), [^{14}C]-L-α-dipalmitoylphosphatidylcholine, and ^{125}I were from PerkinElmer (Cat # NEC141VU250UC, NET139001MC, NEZ033001MC, and NEC682010UC respectively)

Human serum was isolated from plasma from the Houston Methodist Hospital Blood Donor Center by the addition of 20 mM $CaCl_2$ at 37 °C after which the fibrin clot was removed by centrifugation. VLDL, LDL, HDL and lipoprotein-deficient serum (LPDS) were isolated from plasma by sequential flotation at d = 1.006 g/mL, 1.063 g/mL, and 1.21 g/mL as described.[1] Human apo AI was isolated as described.[2] Lipid-free apo AI was labeled as previously described[3] with ^{125}I by N-chloro-benzenesulfonamide immobilized on polystyrene beads (Pierce Iodination Beads, Thermo Scientific, Cat # 28665) according to vendor's instructions and separated from unbound label by gel filtration on PD-10 columns (GE Healthcare Cat# 17-0851-01). [^{125}I]Apo AI was exhaustively dialyzed vs. sterile PBS containing 0.01% EDTA at 4 °C and mixed with unlabeled apo AI to achieve a specific activity of 0.16 µCi/µg. Cells and lipoproteins were analyzed for protein (Bio-Rad DC Protein Assay Kit I, Cat # 5000111) and for phospholipid and FC using enzyme-based kits (Wako Chemicals USA, Inc., Cat # 997-01801 and 993-02501 respectively). Total lipoproteins (TLP; no lipid-free proteins) were obtained by ultracentrifugation (Beckman 50Ti @ 40,000) of whole plasma for 3 d in buffer made d = 1.21 g/mL by the addition of KBr; TLP floats to the top and collected. A His-tagged PLTP, prepared according to Albers,[4] was a gift from Xian-cheng Jiang, Ph.D. (SUNY Downstate Medical Center).

Cell Culture: Baby hamster kidney cells expressing human ABCA1 (BHK-ABCA1) under the control of a mifepristone-inducible promoter, provided by Dr. Ashley Vaughan,[5] were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/100 µg/mL streptomycin at 37 °C in 5% CO2. Prior to nHDL production, cells were plated from a confluent culture at 1:10 dilution into 8-10 T75 flasks/labeling condition, and allowed to attach overnight in the same media at 37°C in 5% CO2. Tissue culture reagents were from InVitrogen (Fisher Thermo Life Sciences).

nHDL Production: Cells were treated with 10 nM mifepristone (Thermo Fisher Scientific Cat # H11001) for 20 h in DMEM/ 0.2% bovine serum albumin (BSA, Sigma-Aldrich Cat # A6003-25G)/100U/mL penicillin/100 µg/mL streptomycin to induce ABCA1 expression and then incubated with 20 µg/mL human apo AI in DMEM/100 U/mL penicillin/100 µg/mL streptomycin for 8 h. Media containing nHDL were collected from multiple T75 flasks, filtered (0.45µm, EMD Millipore, Cat # SCGVU01RE), and centrifuged at 3500 x g for 20 min. The supernatant was concentrated 20-fold using an Amicon Ultracel-10K centrifugal filter (EMD Millipore, Cat # UFC901024). In preliminary experiments, the concentrated nHDL was adjusted to d = 1.28 g/mL by the addition of KBr, overlayed with 2 mL of 1.26g/ml density KBr and floated at 40,000 rpm (Beckman Ti 40 rotor) for 36 hours. The nHDL (~ 1mL) was removed from the top of the tube by pipette. nHDL preparations contained three sizes of nHDL, a minor large (L) nHDL and two major (medium, M and small, S) nHDL species, which were separated by size exclusion chromatography (SEC) over two calibrated Superose HR6 columns (GE HealthCare, Cat # 17-5172-01) in tandem as previously reported.[6] In most experiments, the ultracentrifugation step was omitted and nHDL was concentrated in a single Ultracel-10K filtration step and purified by SEC. The relative amounts of these three species could be altered by loading the cells with FC, as described previously.[7] The most abundant middle-sized nHDL was used for all studies on the in vitro remodeling of nHDL, and for the in vivo studies. nHDL stability was documented by SEC; repeated analytical SEC runs 1 and 3 months apart were identical.

Loading of cells with FC: In some experiments, cells were FC-loaded with a complex of FC and methyl β-cyclodextrin (CDX) for 2 h.[7] Solutions of CDX (2.5 and 5.0 mM; 1320 Da, Sigma-

Aldrich Cat # 4555) were prepared in 300 mL DMEM/ 100 U/ml penicillin/100ug/ml streptomycin at 37°C by mixing for 30 min. Sufficient FC was added to give a CDX/FC molar ratio of 10:1 for 2.5 mM CDX and 8:1 for 5 mM CDX.[7] The FC and CDX were co-solubilized by immersion in a sonic water bath for 3 min and then placed in a rotating water bath at 37°C overnight after which the solutions were filtered (0.45 µm PVDF; EMD Millipore, Cat # SCGVU01RE). After filtration, the 2.5 mM CDX contained 0.06 $\pm$ 0.01 mM FC, and the 5.0 mM CDX contained 0.26 $\pm$ 0.02 mM FC. CDX/FC was added to cells as a 1/10 dilution in DMEM/ 100 U/ml penicillin/100ug/ml streptomycin.

Metabolic Radiolabeling of Cells: Metabolically radiolabeled nHDL-[^{14}C]PL and -[^{3}H]FC was prepared from cells radiolabeled in DMEM/2.5% FBS/100U/mL penicillin/100 µg/mL streptomycin overnight with 0.16 µCi/mL [^{14}C] choline chloride (12 mL media per T75 flask, 1.92 µCi/flask) and/or 0.5 µCi/mL [^{3}H]FC. [^{3}H]FC was added to the media in ~1 µL of ethanol; each flask contained 12 mL media with 6 µCi [^{3}H]FC, (PerkinElmer NET139001MC) as described.[6] This gave BHK-ABCA1 cells labeled with [^{14}C]choline-phospholipids and [^{3}H]FC respectively.

Native and SDS PAGE: The nHDLs were analyzed by non-denaturing PAGE (Life Technologies, Novex, 4–20% Tris-Glycine Gel, Cat # EC6028). nHDL (1–5 µg of protein in 30 µL) were loaded onto gels, and electrophoresed at 125 V for 1.5 h. nHDL (2–8 µg of protein in 30 µL) were also analyzed by SDS PAGE using 4-15% Tris-Glycine Gels (Life Technologies, Novex Cat # EC69028Box) at 170 V for 1.5h. Electrophophoretic bands were visualized with Pierce GelCode Blue Stain Reagent (ThermoFisher, Cat # 24590), destained and recorded on a BioRad Gel Doc Imager. nHDL particle sizes according to SEC and nondenaturing PAGE were determined by comparison with proteins of known size and molecular weight; these were thyroglobulin (17nm, 669 kDa), aldolase (9.6 nm, 158 kDa), ferritin (12.2 nm, 440 kDa), ovalbumin (6.1nm, 44 kDa), and ribonuclease A (3.3nm, 13.7 kDa). Molecular weight standards for the non-denaturing gels were NativeMark™ Unstained Protein Standard Cat# LC0725 (Thermo Fisher) and for the SDS-PAGE gels, SeeBlue™ Plus2 Pre-stained Protein Standard Cat# LC5925 (Thermo Fisher). The SEC calibration was obtained using Gel Filtration Calibration Kits (GE Healthcare Life Sciences, Cat# 28-4038-41, low molecular weight and Cat # 28-4038-42, high molecular weight).

Radiolabeled nHDL and HDL: nHDL-[^{3}H]FC and -[^{14}C]PL for in vitro studies were prepared from cells metabolically labeled with [^{3}H]FC and [^{14}C]PL as above. Alternatively, nHDL-[^{3}H]FC and -[^{14}C]PL for in vitro and in vivo studies was prepared by drying [^{3}H]FC (30 µCi) and [^{14}C]-L-α-dipalmitoylphosphatidylcholine (2.5µCi) onto filter paper (~1x1 cm^{2}), which was incubated with nHDL (480 µg protein) at 37 °C overnight.[8] The resulting specific activities of nHDL-[^{3}H]FC and nHDL-[^{14}C]PC, determined by β-counting a known amount of nHDL-protein, were 57 and 1.0 nCi/µg nHDL respectively; this corresponds to 1.71 x 10^{5} dpm/µg FC and 2.30 x 10^{3} dpm/µg PL. HDL-[^{14}C]PL and -[^{3}H]FC were prepared similarly. nHDL-[^{125}I]apo AI was prepared by a method used previously to prepare HDL-[^{125}I]apo AI[3] by mixing medium sized nHDL (400 µg protein) with [^{125}I]apoAI (40 µg protein). nHDL was separated from free [^{125}I]apo AI by SEC; the [^{125}I] apoAI-positive fractions, which co-eluted with nHDL (A280 nm), were collected. Six percent of the [^{125}I]apo AI incorporated into nHDL. The specific activity of the nHDL-[^{125}I]apo AI was 0.26 µCi/mg nHDL-protein.

nHDL-Sphingomyelin/PL Content: To determine the ratio of [^{14}C]choline labeled phosphatidyl choline (PC) and sphingomyelin (SM) in nHDL-[^{14}C]PL prepared by metabolic labeling with [^{14}C]choline chloride, aliquots of labelled nHDL were added to tubes containing 10 µg each of cold carrier PC and SM and extracted with hexane/2-propanol (3/2 v/v), which was removed under a stream of nitrogen. The residue was extracted twice with chloroform/methanol (1/1, v/v), and the solvent removed under a stream of nitrogen, redissolved in chloroform/methanol (1/1; 20 µL), applied to high performance thin layer chromatography (HPTLC) Silica Gel 60

plates (EMD Chemicals, Darmstadt, Germany, Cat# 1.05748.0001), which were developed with chloroform/methanol/acetic acid/water (65/25/8/4, V/V) to ~80-90% of plate length. Distribution of radioactivity into PC and SM was quantified on a Typhoon Image Analyzer (GE HealthCare). The ratio of [^{14}C]choline labeled PC and SM in whole cells was determined similarly, using aliquots of the cell total lipid extract.

Interaction of nHDL with Serum Lipoproteins: nHDL containing [^{3}H]FC, [^{14}C]PL, or [^{125}I]apo AI prepared as described above were incubated with serum for various times and fractionated by SEC, which separated HDL, low- and very low-density lipoproteins (LDL, VLDL) into fractions that were collected.[9] Similar experiments were conducted with isolated lipoproteins.

LCAT Activity vs. nHDL and HDL: nHDL- and HDL-[^{3}H]FC (100 µL, 0.27 mg/mL) were transferred to mouse plasma (1 mL) at 37 °C. At various times aliquots (50 µL) were transferred to methanol (1 mL), extracted into hexane:2-propanol (1ml, 3:2, v/v), and centrifuged at low speed for 30 min. The clear upper phase was transferred to a glass conical tube and the solvent reduced under a stream of nitrogen. The dried lipid extract was dissolved in chloroform/methanol (20 µL; 1:1 v/v) and applied to HPTLC Silica Gel 60 plates with cold carrier (FC and CE), and developed in hexane:ethyl acetate (4:1, v/v) to ~ 80% of plate length. The FC- and CE-positive spots were visualized by exposure to iodine vapor, transferred to scintillation vials, and β-counted. LCAT activity was expressed as percent FC converted to CE/min.

Kinetics of nHDL-[^{3}H]FC Transfer to LDL: The kinetics of nHDL-[^{3}H]FC desorption were determined as described.[10,11] nHDL (0.54 µg/mL) and LDL (18 µg/mL) in TBS were incubated for various times at 37 °C in Eppendorf tubes, which were placed on wet ice for 30 sec and mixed with heparin-Mn^{+2}. The LDL-heparin-Mn^{+2} complex was sedimented at 14,000 x g (4 °C) for 5 min after which an aliquot of the supernatant was collected and β-counted. The rate of nHDL-[^{3}H]FC desorption and transfer to LDL was determined by fitting the data—supernatant counts vs. time—to a 2-parameter polynomial (SigmaPlot) from which a rate constant (k) was obtained; $t_{1/2}$ was calculated as 0.69/k.

In vivo nHDL Metabolism: C57BL/6J mice (Jackson Laboratories) were retro-orbitally injected with nHDL labeled with [^{3}H]FC (0.18 µCi), [^{14}C]PL (0.008 µCi), and [^{125}I]apo AI (0.030 µCi), with 3 -4 mice per time point, and euthanized at various times post-injection. The blood and major organs were collected. In **Figure 6** a total of 25 mice were used, 10 males and 15 females. In **Supplementary Figure VIII,** a total of 19 mice, 10 male and 9 females were used. Blood was centrifuged to collect plasma and red blood cells. Aliquots of organs were weighed and β- and/or γ-counted. Total plasma volume was calculated as 3.5% of body weight, and blood volume was calculated as BV = 0.06 x body weight (g) + 0.77.[12] Organ radioactivity was expressed as the total radioactivity per organ. In vivo kinetic data for the plasma decay of nHDL-[^{125}I]apo AI were fitted to a four-parameter bi-exponential function; kinetic constants for [^{3}H]FC and [^{14}C]PL were obtained from a three parameter exponential function. Hepatic nHDL accretion was determined by fitting the rising initial slope of radiolabel vs. time to a second-order polynomial. For comparison, in vivo metabolism of HDL labeled with [^{3}H]FC and [^{14}C]PL was analyzed in the same manner. All kinetic analyses were performed in SigmaPlot (Systat Software, Inc).

nHDL- and HDL-[^{3}H]FC and [^{14}C]PL Uptake by Huh7 Cells: Uptake of [^{3}H]FC and [^{14}C]PL from nHDL and HDL by Huh7 cells was assayed as previously described.[13] Briefly, PLTP (10 µg/mL) or buffer was added to confluent Huh7 cells in serum-free media containing 0.5% fatty acid free BSA. Cellular uptake was initiated by addition of nHDL- or HDL- [^{3}H]FC and [^{14}C]PL (20 µg/mL protein); media and cells were collected at various times. Cell-lipid was extracted with 2-propanol. DPM in media and cell-lipids were determined by β-counting. Input nHDL and HDL

respectively contained 12,156 and 12,950 DPM [^{3}H]FC and 3,126 and 2,794 DPM [^{14}C]PL in 20 µg lipoprotein; data are expressed as cell-associated DPM per mg-cell protein. Slopes were compared and p-values obtained using Graph Pad Prism 7.02.

Statistical Analyses: Data were fit to the appropriate kinetic equations using SigmaPlot as noted in the figure legends. The linear regression slopes in **Figure 7** were tested for significant differences using Graph Pad Prism 7.02 Software. Data were collected in triplicate or greater and expressed as means ± SD; differences with a p-value <0.05 were considered significant.

REFERENCES

1. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest.* 1955;34:1345-1353.
2. Brewer HB, Jr., Ronan R, Meng M, Bishop C. Isolation and characterization of apolipoproteins A-I, A-II, and A-IV. *Methods Enzymol.* 1986;128:223-246.
3. Gillard BK, Bassett GR, Gotto AM, Jr., Rosales C, Pownall HJ. Scavenger receptor B1 (SR-B1) profoundly excludes high density lipoprotein (HDL) apolipoprotein AII as it nibbles HDL-cholesteryl ester. *J Biol Chem.* 2017;292:8864-8873.
4. Albers JJ, Day JR, Wolfbauer G, Kennedy H, Vuletic S, Cheung MC. Impact of site-specific N-glycosylation on cellular secretion, activity and specific activity of the plasma phospholipid transfer protein. *Biochim Biophys Acta.* 2011;1814:908-911.
5. Vaughan AM, Oram JF. ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. *J Lipid Res.* 2003;44:1373-1380.
6. Lyssenko NN, Nickel M, Tang C, Phillips MC. Factors controlling nascent high-density lipoprotein particle heterogeneity: ATP-binding cassette transporter A1 activity and cell lipid and apolipoprotein AI availability. *FASEB J.* 2013;27:2880-2892.
7. Patton S, Benson AA. Synthesis of phytanic acid. *J Lipid Res.* 1966;7:452-453.
8. Schwartz CC, Halloran LG, Vlahcevic ZR, Gregory DH, Swell L. Preferential utilization of free cholesterol from high-density lipoproteins for biliary cholesterol secretion in man. *Science.* 1978;200:62-64.
9. Pownall HJ, Hosken BD, Gillard BK, Higgins CL, Lin HY, Massey JB. Speciation of human plasma high-density lipoprotein (HDL): HDL stability and apolipoprotein A-I partitioning. *Biochemistry.* 2007;46:7449-7459.
10. Lund-Katz S, Hammerschlag B, Phillips MC. Kinetics and mechanism of free cholesterol exchange between human serum high- and low-density lipoproteins. *Biochemistry.* 1982;21:2964-2969.
11. Davidson WS, Heink A, Sexmith H, et al. The effects of apolipoprotein B depletion on HDL subspecies composition and function. *J Lipid Res.* 2016;57:674-686.
12. Connelly LM. Pilot studies. *Medsurg Nurs.* 2008;17:411-412.
13. Gillard BK, Rodriguez PJ, Fields DW, et al. Streptococcal serum opacity factor promotes cholesterol ester metabolism and bile acid secretion in vitro and in vivo. *Biochim Biophys Acta.* 2016;1861:196-204.