Influence of apolipoprotein A-I and apolipoprotein A-II availability on nascent HDL heterogeneity

Eric T. Alexander and Michael C. Phillips[1]

GI/Nutrition/Hepatology Division, The Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104

Abstract **It is important to understand HDL heterogeneity because various subspecies possess different functionalities. To understand the origins of HDL heterogeneity arising from the existence of particles containing only apoA-I (LpA-I) and particles containing both apoA-I and apoA-II (LpA-I+A-II), we compared the abilities of both proteins to promote ABCA1-mediated efflux of cholesterol from HepG2 cells and form nascent HDL particles. When added separately, exogenous apoA-I and apoA-II were equally effective in promoting cholesterol efflux, although the resultant LpA-I and LpA-II particles had different sizes. When apoA-I and apoA-II were mixed together at initial molar ratios ranging from 1:1 to 16:1 to generate nascent LpA-I+A-II HDL particles, the particle size distribution altered, and the two proteins were incorporated into the nascent HDL in proportion to their initial ratio. Both proteins formed nascent HDL particles with equal efficiency, and the relative amounts of apoA-I and apoA-II incorporation were driven by mass action. The ratio of lipid-free apoA-I and apoA-II available at the surface of ABCA1-expressing cells is a major factor in determining the contents of these proteins in nascent HDL. Manipulation of this ratio provides a means of altering the relative distribution of LpA-I and LpA-I+A-II HDL particles.**—Alexander, E. T., and M. C. Phillips. **Influence of apolipoprotein A-I and apolipoprotein A-II availability on nascent HDL heterogeneity.** ***J. Lipid Res.*** **2013.** 54: **3464–3470.**

Supplementary key words ATP binding cassette A1 • cholesterol • lipoprotein • phospholipid

High density lipoprotein (HDL) cholesterol levels in plasma are inversely associated with the risk of cardiovascular disease (1). This antiatherogenic behavior is thought to arise in part from the central role of HDL in reverse cholesterol transport (RCT), the pathway by which excess cholesterol is removed from peripheral tissues and transported to the liver for excretion from the body (2–5). The current consensus is that the structure and composition of HDL particles, not just the plasma HDL cholesterol levels, are important for the efficiency of the RCT pathway and the inverse relationship with the incidence of atherosclerosis. Plasma HDL comprises a heterogeneous assembly of different size particles, with variations in lipid and protein content (6, 7). Apolipoprotein (apo)A-I and apoA-II are the major protein constituents, comprising 70% and 20% of total HDL protein, respectively (8). ApoA-I has received the most attention to this point, and its structure-function relationship in preventing atherosclerosis is relatively well understood (7). In comparison, less attention has been paid to apoA-II. Human apoA-II is an amphipathic protein synthesized and secreted by the liver and small intestine as an 82-amino acid proprotein that is proteolytically cleaved to the mature 77-amino acid protein (9). The concentration of apoA-II in plasma is 31 ± 6 mg/dl, much lower than the apoA-I level (123 ± 28 mg/dl) (10). ApoA-II circulates as a 17.4 kDa homodimer formed by two mature apoA-II molecules linked by a disulphide bond at residue 6 (11).

HDL heterogeneity originates at the point of biogenesis at which apoA-I and ABCA1 interact to create discoidal nascent particles; the relative available lipid/apoA-I ratio controls the size distribution (12). The heterogeneity of mature spherical HDL is also affected by remodeling by lipases and lipid transfer proteins in the plasma (6). It is important to understand HDL heterogeneity because different HDL subspecies exhibit different functionalities (13). The existence of LpA-I and LpA-I+A-II particles is another aspect of HDL heterogeneity that influences HDL functionality. For instance, LpA-I+A-II HDL is less effective than LpA-I HDL at promoting selective cholesteryl ester uptake via SR-B1 (14) and the transfer of cholesteryl ester by CETP (15). Also, compared with LpA-I particles, LpA-I+A-II particles are less effective at promoting cholesterol esterification via LCAT (16), although the abilities of the two types of HDL to promote cellular cholesterol efflux are similar (17, 18).

To better understand the origins of nascent HDL heterogeneity arising from the coexistence of LpA-I and LpA-I+A-II particles, we examined the particles formed when

This work was supported National Institutes of Health Grant HL-22633.

Manuscript received 14 August 2013 and in revised form 18 September 2013.

Published, JLR Papers in Press, October 1, 2013
DOI 10.1194/jlr.M043109

[1]To whom correspondence should be addressed.
e-mail: phillipsmi@email.chop.edu

both apoA-I and apoA-II are available to ABCA1 on the surface of HepG2 cells, which were used because the liver is the primary source of nascent HDL in vivo (19).

MATERIALS AND METHODS

Cell culture and biogenesis of nascent HDL

HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA). The cell line was maintained in Eagles minimal essential medium (MEM) supplemented with fetal bovine serum (FBS) to 10% and gentamicin to 50 μg/ml at 37°C in 5% CO_2. To study nascent HDL particle formation, cells from a confluent culture were plated at a 1/5 dilution (unless specified otherwise) in three or four T75 flasks per condition, allowed to grow for 4–5 days in the above medium, and then labeled overnight with 0.5 μCi/ml [1,2-^{3}H(N)]cholesterol (radiochemicals were from PerkinElmer, Waltham, MA). The cells were then treated with 9-*cis*-retinoic acid (10 μM) and 22-hydroxy-cholesterol (10 μM) for 16–20 h in MEM/0.2% BSA (BSA; fraction V, fatty acid free, EMD Millipore, Billerica, MA)/50 μg/ml gentamicin and CP113818 (ACAT inhibitor, 2μg/ml) (Sandoz). These cells in which ABCA1 activity was upregulated were exposed to 0–40 μg/ml of either human apoA-I, human apoA-II, human [^{14}C]apoA-I [trace labeled to ~1 μCi/mg as previously described (20)], and/or human [^{3}H]apoA-II in MEM/50 μg/ml gentamicin plus ACAT inhibitor (2 μg/ml) for 2–8 h. ApoA-I and apoA-II were isolated from frozen plasma of normolipidemic donors as described earlier (21, 22). Cell medium containing nascent HDL particles was collected, filtered through a 0.45 μm PVDF membrane filter unit (EMD Millipore, Billerica, MA), reduced in volume 20× from 30 ml to 1.5 ml using an Amicon Ultracel-10K centrifugal filter (EMD Millipore, Billerica, MA) and stored at 4°C for further analysis (12, 20, 23).

Cell cholesterol efflux assay

HepG2 cells were seeded in 24-well plates at a 1/10 dilution from a confluent culture, allowed to attach overnight, labeled with 0.5 μCi/ml [1,2-^{3}H(N)]cholesterol overnight, and upregulated for ABCA1 expression for 16–20 h as described above. The cells were then exposed to 10 μg/ml of either human apoA-I or apoA-II in MEM/50 μg/ml gentamicin for 4 h. The medium was collected and filtered through a 96-well filter plate (EMD Millipore, Billerica, MA); the radioactivity in a 100 μl aliquot of each sample was determined using a scintillation counter (23). Cell lipids were extracted with hexane-isopropanol (3:2, v/v), the solvent was evaporated, the lipids were dissolved in Scintiverse, and the radioactivity was determined with a scintillation counter. The percentage of cellular cholesterol released to apoA-I was calculated by dividing the [^{3}H] counts in the medium by the sum of [^{3}H] counts in the medium and cells and multiplying by 100.

Gel filtration chromatography of nascent HDL

A 1 ml aliquot of the 20× concentrated cell medium containing nascent HDL was resolved into 1 ml fractions on a calibrated HiLoad 16/60 Superdex 200 gel filtration column (GE Healthcare, Mickleton, NJ) using tris-buffered saline (TBS), pH 7.4, as the mobile phase. Each fraction was combined with 5 ml of ScintiVerse BD cocktail (Fisher Scientific, Pittsburg, PA) and read in a scintillation counter; when dual labeling was used, [^{3}H] and [^{14}C] counts were adjusted for the energy emission spectra overlap. Alternatively, the fractions containing the larger (>9 nm) or the smaller (<9 nm) nascent HDL particles were combined, concentrated to 0.8–1.0 ml using Amicon Ultracel-10K centrifugal filter units, and stored at 4°C for further analysis. The gel filtration column was washed between runs with 30% isopropanol and 1 M NaOH, as recommended by the manufacturer. The following standards (Sigma-Aldrich, St. Louis, MO) were used to calibrate the column: cytidine, (total volume, V_t); thyroglobulin, 17.0 nm; apoferritin, 12.2 nm; lactic dehydrogenase, 8.16 nm; BSA, 7.1 nm; carbonic anhydrase, 4.4 nm; blue dextran, (void volume, V_o). The particles sizes corresponding to the various fractions were determined by comparing their K_{av} values with those of the above proteins (20). K_{av} was calculated using the following equation: $K_{av} = (V_e - V_0) / (V_t - V_0)$, where V_e is the elution volume of the HDL fraction. The apparent particle size [hydrodynamic diameter (d) in nanometers] was derived from the following equation: $\log_{10} d = -1.103\ K_{av} + 1.372$. An increase in V_e of 1 ml corresponded to an increase in d of 0.7 nm.

HDL separation into LpA-I and LpA-I+A-II

As described earlier (24–26), chromatography on activated thiol-sepharose beads was used to separate LpA-I and LpA-I+A-II particles. In brief, an HDL sample was reduced in TBS with 10 mM dithiothreitol (DTT) and then dialyzed into TBS containing 1 mM DTT overnight. Thiopropyl-sepharose beads (0.25 g) were stirred into deionized water for 15 min and then washed extensively. The washed beads were resuspended in 1 ml of TBS and divided into two equal aliquots. One aliquot was stirred with the reduced HDL, and the other was transferred to a 1 × 6 cm chromatography column. After 2 h, the HDL slurry mixture was added to the column and allowed to settle. The unbound LpA-I was eluted with TBS and collected. The column was then washed with 30 ml of TBS. After this treatment, LpA-I+A-II was eluted by the addition of 20 mM DTT to the TBS (10 ml). The distributions of apoA-I and apoA-II were confirmed by scintillation counting to detect both ^{14}C-apoA-I and ^{3}H-apoA-II.

RESULTS

Influence of ABCA1 upregulation on cholesterol efflux from HepG2 cells

HepG2 cells were plated, allowed to reach confluency, and labeled with [1,2-^{3}H(N)]cholesterol, and then half was treated with 9-*cis*-retinoic acid and 22-hydroxy-cholesterol for 18 h to upregulate ABCA1. Fresh medium without an apolipoprotein acceptor was then added to both sets of cells and incubated for 4 h. The efflux of cholesterol from nonupregulated cells was 1.9 ± 0.6% over 4 h, whereas the efflux from upregulated cells was 3.2 ± 0.3% over 4 h. Efflux with the latter cells was carried out for 24 h to generate sufficient HDL to allow FPLC analysis (**Fig. 1**). These HepG2 cells secreted nascent HDL particles that contained endogenously synthesized apoA-I and apoA-II as reported previously (27–30). The efflux medium was also analyzed by immunoblotting; the ratio of apoA-I to apoA-II was approximately 4/1 w/w (data not shown). The nascent HDL particles produced by HepG2 cells are heterogeneous (30). As shown in Fig. 1, when ABCA1 is upregulated, the cholesterol released from the cell is incorporated into two populations of nascent HDL particles. The larger particles elute from the Superdex 200 column with a peak maximum of V_e = 63 ml, and the smaller

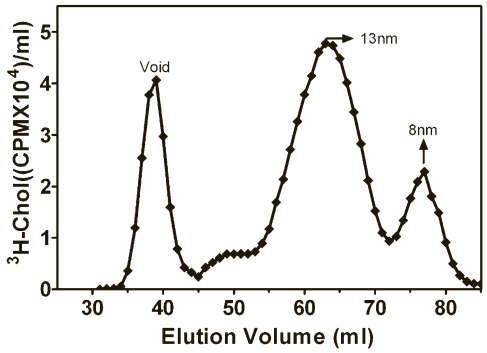

Fig. 1. Production of nascent HDL by HepG2 cells. HepG2 cells were grown to near confluence, labeled with ^{3}H-cholesterol, and then ABCA1 expression was upregulated. Fresh media with no exogenous apolipoproteins was added and cholesterol efflux was allowed to continue for 24 h. Efflux media was collected, filtered, concentrated, and then run over a Superdex 200 column. The radioactivity in 1 ml fractions of the elution buffer was determined.

particles elute at V_e = 77 ml; these peak positions correspond to particle hydrodynamic diameters of 13 and 8 nm, respectively.

Cholesterol efflux from HepG2 cells in the presence of exogenous apoA-I and apoA-II

We performed comparative experiments to assess the abilities of exogenous lipid-free apoA-I (molecular weight 28016) and apoA-II (molecular weight in the dimerized state 17414) to mediate efflux of cholesterol from HepG2 cells in which ABCA1 was upregulated to enhance production of nascent HDL particles. ApoA-I and apoA-II were added to the media at a concentration of 2.5 μg/ml and incubated with the cells for 2, 4, and 6 h. There were no differences between apoA-I and apoA-II in the rate of cholesterol efflux over the 6 h incubation period (**Fig. 2A**). The initial rate of cholesterol efflux over the first 4 h to apoA-I was 2.9 ± 0.15% per h and 3.2 ± 0.15% per h to apoA-II. To address the effect of protein concentration, either apoA-I or apoA-II was added to the efflux media in the range of 2.5–20 μg/ml. Fig. 2B shows that apoA-I and apoA-II were similarly efficient at promoting cholesterol efflux with V_{max} (percentage cholesterol efflux over 4 h) values of 9.8 ± 1.1 and 9.5 ± 1.3 respectively, and K_m (micrograms of apo per milliliter) values of 1.9 ± 1.0 and 1.5 ± 0.9, respectively. The catalytic efficiencies (V_{max} / K_m) were similar to values for ABCA1-mediated cholesterol efflux in other cell types (31, 32).

Comparison of nascent HDL formed by apoA-I and apoA-II

Although apoA-I and apoA-II promote similar cholesterol efflux from HepG2 cells, it was important to compare the nascent HDL particles formed by the two proteins. To this end, conditioned efflux media containing either apoA-I or apoA-II were analyzed by gel filtration chromatography (**Figs. 3 and 4**). FPLC traces of both ^{14}C-apoA-I and ^{3}H-cholesterol (Fig. 3) showed two HDL peaks with maxima at V_e = 66 and 76 ml, respectively. The hydrodynamic diameters of these LpA-I HDL particles deduced

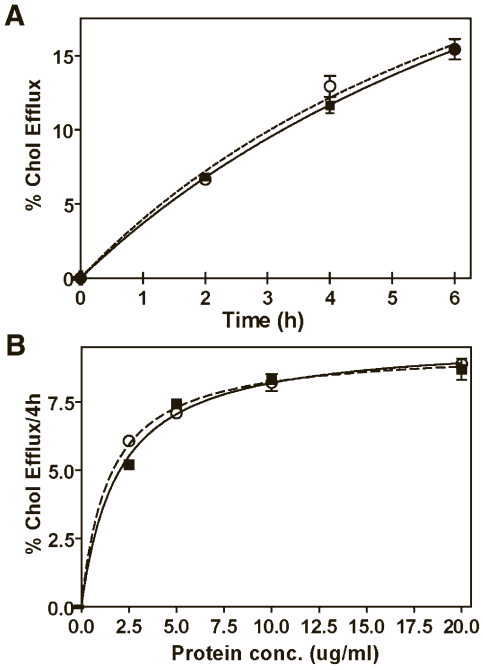

Fig. 2. Time and protein concentration dependence of ABCA1-mediated cholesterol efflux from HepG2 cells induced by apoA-I and apoA-II. HepG2 cells were grown to near confluence, labeled with ^{3}H-cholesterol, and then ABCA1 expression upregulated. (A) Cells were incubated with 2.5 μg/ml of apoA-I (filled squares) or apoA-II (open circles) for the indicated times. (B) Cells were incubated with indicated concentrations of apoA-I (filled squares) or apoA-II (open circles) for 4 h, and the resulting data were fitted to the Michaelis-Menten equation.

from the peak V_e values were 12 and 8 nm, which agrees with a prior report for HepG2 cells treated similarly (33). Efflux to apoA-II gave rise to a ^{14}C-cholesterol FPLC profile with one broad peak at V_e = 67 ml (Fig. 4). The equivalent ^{3}H-apoA-II profile had peaks with V_e values of 67, 71, and 81 ml, corresponding to LpA-II particle diameters of 11, 10, and 7 nm.

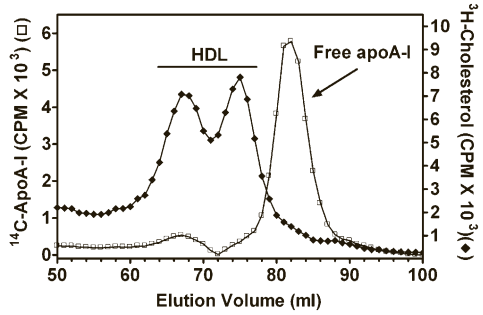

Fig. 3. Profile of nascent HDL containing apoA-I (LpA-I). HepG2 cells were grown to near confluence, labeled with ^{3}H-cholesterol, and treated with 9-cis-retinoic acid and 22-hydroxycholesterol. ABCA1-mediated cholesterol efflux was induced by adding 10 μg/ml of apoA-I. Efflux media was collected after 6 h, filtered, concentrated, and then run over a Superdex 200 column. The radioactivity in 1 ml fractions of the elution buffer was determined. ApoA-I (open squares) cholesterol (filled diamonds).

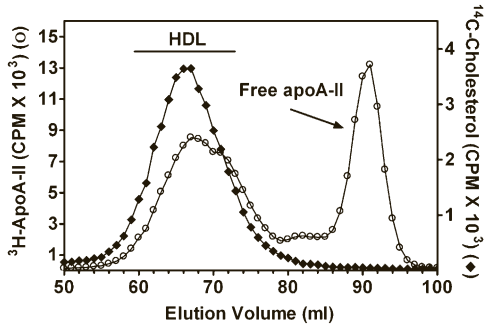

Fig. 4. Profile of nascent HDL containing apoA-II (LpA-II). HepG2 cells were prepared as described in Fig. 3. ABCA1-mediated cholesterol efflux was induced by adding 10 μg/ml of apoA-II, and the conditioned medium was analyzed as described in Fig. 3. ApoA-II (open circles) cholesterol (filled diamonds).

Nascent HDL produced by mixtures of apoA-I and apoA-II

To determine what types of HDL particles are generated when apoA-I and apoA-II are present in equal amounts, efflux medium with 5 μg/ml of both apoA-I and apoA-II was analyzed by gel filtration chromatography (**Fig. 5**). The ^{14}C-apoA-I profile (Fig. 5A) had one well-defined peak with a maximum V_e at ~66 ml and a small peak centered around 75 ml, which preceded the lipid-free apoA-I peak at ~81 ml. The corresponding ^{3}H-apoA-II profile had one large, broad peak with a maximum V_e at ~66 ml and a second, smaller peak with a maximum at 80 ml. The gel filtration profile from a separate efflux

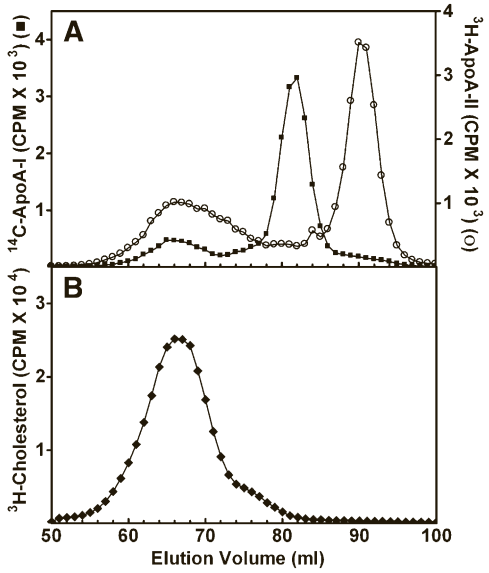

Fig. 5. Profile of nascent HDL containing a mixture of apoA-I and apoA-I (LpA-I+A-II). HepG2 cells were prepared as described in Fig. 3, except that some cells were not labeled with ^{3}H-cholesterol. ABCA1-mediated cholesterol efflux was induced by adding a mixture containing 5 μg/ml of apoA-I and 5 μg/ml of apoA-II. The conditioned medium was analyzed as described in Fig. 3. (A) A mixture of 5 μg/ml of ^{14}C-apoA-I and 5 μg/ml ^{3}H-apoA-II was incubated with cells containing unlabeled cholesterol. (B) A mixture containing 5 μg/ml each of unlabeled apoA-I and apoA-II was incubated with ^{3}H-cholesterol-labeled cells. ApoA-I, filled squares; ApoA-II, open circles; cholesterol, filled diamonds.

experiment using media containing 5 μg/ml each of unlabeled apoA-I and apoA-II and cells that had been labeled with ^{3}H-cholesterol showed one peak with a maximum V_e at ~66 ml and a noticeable shoulder centered at 76 ml (Fig. 5B). The hydrodynamic diameter of the primary HDL peak at V_e = 66 ml, as indicated by all three FPLC profiles, was 12 nm. The hydrodynamic diameters of the particles in the secondary HDL peaks were 8 and 7 nm. Samples of nascent HDL formed with apoA-I, apoA-II, or a mixture of the two proteins that were purified from conditioned medium were analyzed by negative stain electron microscopy and shown to contain discoidal HDL particles that were apparent in the electron micrographs as characteristic rouleaux (data not shown).

The presence of both apoA-I and apoA-II in the efflux media resulted in a shift in the size distribution of the nascent HDL particles, reflecting formation of HDL species containing both apoA-I and apoA-II (LpA-I+A-II) (see Figs. 3, 4, and 5A). A similar effect on HDL size occurs in mice transgenic for both human apoA-I and apoA-II (34). To investigate this phenomenon in more detail, the molar ratio of ^{14}C-apoA-I to ^{3}H-apoA-II incubated with the HepG2 cells was varied from 1:1 to 16:1. The distributions of the two proteins between the various HDL species are summarized in **Table 1**. It is apparent that apoA-I and apoA-II were incorporated into the nascent HDL particles in proportion to their levels in the original mixture incubated with the HepG2 cells. As shown in Figs. 3 and 4, the size distributions of nascent LpA-I and LpA-II HDL particles were different, and as expected, the size distribution of the particles created from a mixture of apoA-I and apoA-II reflected the presence of both proteins. Thus, as shown in **Fig. 6**, the peak distribution of apoA-II in LpA-II particles was at V_e = 71 ml (Fig. 6B), and the proportion of apoA-II eluting at this position was progressively decreased as apoA-II was mixed with increasing amounts of apoA-I (Fig. 6C, D). The addition of apoA-I led to formation of LpA-I+A-II particles that eluted at V_e = 67 ml, causing the maximal distribution of apoA-II to shift to this position (Fig. 6C, D), which corresponds to the V_e of nascent LpA-I particles (Fig. 6A).

Given the extensive overlap in gel filtration FPLC profiles of LpA-I and LpA-II nascent HDL separated on the basis of particle size, it was difficult to resolve any LpA-I+A-II particles that formed at various initial molar ratios of

TABLE 1. Effect of initial apoA-I:apoA-II ratio on HDL composition

Initial ApoA-I/ApoA-II Molar Ratio[a]	ApoA-I:ApoA-II Molar Ratio of HDL[b]
0.6	1.0 ± 0.1
1	1.0 ± 0.1
2	1.9 ± 0.4
4	3.7 ± 1.0
8	9.4 ± 2.3
16	13.7 ± 3.3

[a] Initial ratio of ^{14}C-labeled apoA-I to ^{3}H-labeled apoA-II in the efflux media applied to upregulated HepG2 cells.

[b] Ratio of ^{14}C-apoA-I to ^{3}H-apoA-II in 11 individual HDL fractions (elution volume 60–71 ml) of FPLC profile (e.g., Fig. 5A). Average of two separate experiments, with the exception of 0.6 and 1 ratios, which were performed once each. Mean ± SD (n = 11–22).

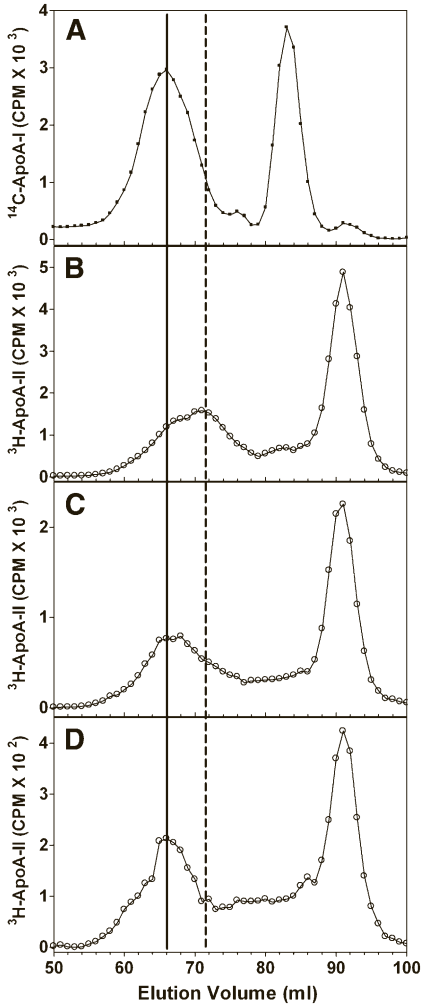

Fig. 6. Profile of nascent HDL generated at different initial molar ratios of apoA-I and apoA-II. HepG2 cells were prepared as described in Fig. 3, except that the cells were not labeled with ^{3}H-cholesterol. ABCA1-mediated cholesterol efflux was induced by adding apoA-I and apoA-II. The conditioned medium was analyzed as described in Fig. 3. (A) 10 μg/ml of ^{14}C-apoA-I. (B) 10 μg/ml ^{3}H-apoA-II. (C) 1:1 mol:mol mixture of ^{14}C-apoA-I and ^{3}H-apoA-II. (D) 4:1 mol:mol mixture of ^{14}C-apoA-I and ^{3}H-apoA-II. For clarity, only the ^{3}H-apoA-II trace is shown in (C) and (D). The solid vertical line at an elution volume of 66 ml corresponds to the LpA-I peak in (A), and the dashed line V_e = 71 ml corresponds to the maximum for LpA-II in (B).

apoA-I and apoA-II. To solve this problem, the nascent HDL was separated into LpA-I and LpA-I+A-II fractions by covalent chromatography using thiopropyl-sepharose. Analysis of three separate samples of nascent HDL containing an average apoA-I-to-apoA-II molar ratio of 2:1 demonstrated that 84 ± 5% of the total apoA-I resided on HDL particles that did not bind to the column. These particles contained an average apoA-I-to-apoA-II dimer molar ratio of 5 to 1. In contrast, the 16 ± 5% of apoA-I in HDL particles that bound to the thiopropyl-sepharose column and were subsequently eluted with DTT were present in apoA-II-enriched particles (1/4 mol/mol apoA-I/apoA-II). It is apparent that a nascent HDL preparation with an average apoA-I/apoA-II mol ratio of 2/1 comprises individual particles containing very different relative amounts of the two proteins.

DISCUSSION

Plasma HDL consists of a diverse population of particles that have significant differences in composition and size. It is important to understand this heterogeneity because different HDL subspecies exhibit different biological activities in vivo, and understanding their biogenesis is the first step in understanding their functionalities (6, 13) and identifying subspecies with desired cardioprotective properties. Nascent HDL particle heterogeneity is influenced by the ratio of cell lipids made available by ABCA1 activity to available apoA-I (12) and by the composition of the available cell lipids (35). Another potential source of nascent HDL heterogeneity is the composition of the available apolipoproteins during HDL biogenesis. To better understand the origins of nascent HDL heterogeneity arising from the existence of LpA-I and LpA-I+A-II particles, we examined the particles formed when both apoA-I and apoA-II are available to ABCA1 on the surface of HepG2 cells.

Before analyzing the nascent HDL particles produced by apoA-I and apoA-II, we first quantified the cholesterol efflux from HepG2 cells elicited by the addition of exogenous apoA-I and apoA-II. There were no differences in the rate of cholesterol efflux over time (Fig. 2A) or with the effect of protein concentration (Fig. 2B) between apoA-I and apoA-II. Given the increased hydrophobicity of apoA-II (36, 37) and the somewhat enhanced ability of apoA-II to solubilize dimyristoyl phosphatidylcholine vesicles compared with apoA-I (38), we had anticipated that apoA-II may be a better acceptor of ABCA1-mediated cholesterol efflux from HepG2 cells. However, despite the differences in physical properties, apoA-I and apoA-II promote cholesterol efflux equally efficiently.

Although apoA-I and apoA-II promoted ABCA1-mediated cholesterol efflux similarly, the resulting LpA-I and LpA-II nascent HDL particles had somewhat different size distributions (Figs. 3 and 4). Presumably, this variation is a consequence of the two proteins of different size requiring different interfacial areas to be optimally packed and cover the phospholipid acyl chains at the edge of the discoidal HDL particles (39, 40). Both proteins adopt a belt-like molecular arrangement around the edge of the segment of phospholipid bilayer in such particles (39). When both apoA-I and apoA-II were present, the distribution of nascent HDL subspecies produced was altered, consistent with a mixture of LpA-I and LpA-I+A-II particles being produced. It is apparent that both proteins are incorporated into nascent HDL in proportion to their availability as lipid-free proteins at the surface of the ABCA1-expressing HepG2 cells (**Table 1**). ABCA1 activity creates two types of apolipoprotein binding sites at the cell surface (41). Direct apolipoprotein/ABCA1 interaction creates a low capacity site that serves a regulatory role, and apolipoprotein/membrane lipid interactions create a much higher capacity site that is involved in the assembly of nascent

HDL particles. The latter site is an exovesiculated domain of plasma membrane lipids to which apoA-I and apoA-II can bind (42). The phospholipid bilayer in such a domain is destabilized by the penetration of apolipoprotein amphipathic α-helices (22), leading to solubilization and formation of discoidal nascent HDL particles. When both apoA-I and apoA-II are present, the two proteins bind simultaneously and become incorporated into the HDL particles formed. The relative degree of binding of apoA-I and apoA-II to the membrane lipid surface is driven by mass action effects, which explains why the degree of incorporation of the two proteins into HDL particles is in proportion to their availability (on a molar basis). Consequently, the apoA-I/apoA-II ratio in nascent HDL particles at the moment of formation is controlled by the relative concentrations of the two lipid-free proteins at the HepG2 cell surface where ABCA1 is active. The relative apoA-I/apoA-II content of these particles can then modified by subsequent HDL remodeling events due to the activity of plasma factors such as LCAT (43). In our experiments, the lipid-free apoA-I and apoA-II were added exogenously to the exterior of the HepG2 cells. However, the process of mass-action driven incorporation of the two proteins into nascent HDL particles is of physiological relevance because HepG2 cells (29, 44–50) and hepatocytes (51, 52) secrete significant proportions of apoA-I and apoA-II as lipid-free proproteins that are rapidly converted to the lipid-free mature proteins.

Although the overall degree of incorporation of apoA-I and apoA-II into nascent HDL particles when both proteins are present is driven by mass action effects, the apoA-I/apoA-II ratio in individual HDL particles can diverge significantly from the population average. Thus, HDL fractions containing apoA-I/apoA-II molecular ratios of 5/1 and 1/4 were separated by thiol-affinity chromatography from a nascent HDL preparation with an average apoA-I/apoA-II molar ratio of 2/1. It follows that a given size of nascent HDL particle can contain different ratios of apoA-I and apoA-II. Consistent with this finding, alterations of the apoA-I/apoA-II ratio in either discoidal or spherical HDL particles can have little effect on particle size (15, 53–55). Furthermore, Segrest and colleagues recently isolated individual HDL subspecies using immunoaffinity chromatography and identified seven populations with different stoichiometries of apoA-I and apoA-II, many of which were similar in size (40).

In summary, the present comparison of nascent HDL formation with apoA-I and apoA-II revealed the following findings: *i*) ApoA-I and apoA-II promoted similar levels of ABCA1-mediated cholesterol efflux, but the resulting nascent HDL particles were of different sizes; *ii*) both proteins formed nascent HDL with equal efficiency, and the relative amounts of apoA-I and apoA-II incorporation were driven by mass action; *iii*) LpA-I+A-II HDL particles of a given size can contain different ratios of apoA-I and apoA-II; and *iv*) the ratio of available apoA-I and apoA-II at the cell surface is a major factor in determining the contents of these proteins in nascent HDL. Manipulation of this ratio may provide a novel approach to altering the LpA-I/LpA-I+A-II HDL distributions and to potentially generating HDL populations with enhanced cardioprotective characteristics.

REFERENCES

1. Lewis, G. F., and D. J. Rader. 2005. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ. Res.* **96:** 1221–1232.
2. Yancey, P. G., A. E. Bortnick, G. Kellner-Weibel, M. De La Llera-Moya, M. C. Phillips, and G. H. Rothblat. 2003. Importance of different pathways of cellular cholesterol efflux. *Arterioscler. Thromb. Vasc. Biol.* **23:** 712–719.
3. Curtiss, L. K., D. T. Valenta, N. J. Hime, and K. A. Rye. 2006. What is so special about apolipoprotein AI in reverse cholesterol transport? *Arterioscler. Thromb. Vasc. Biol.* **26:** 12–19.
4. Rader, D. J., E. T. Alexander, G. L. Weibel, J. Billheimer, and G. H. Rothblat. 2009. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *J. Lipid Res.* **50(Suppl):** S189–S194.
5. Rosenson, R. S., H. B. Brewer, Jr., W. S. Davidson, Z. A. Fayad, V. Fuster, J. Goldstein, M. Hellerstein, X. C. Jiang, M. C. Phillips, D. J. Rader, et al. 2012. Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. *Circulation.* **125:** 1905–1919.
6. Camont, L., M. J. Chapman, and A. Kontush. 2011. Biological activities of HDL subpopulations and their relevance to cardiovascular disease. *Trends Mol. Med.* **17:** 594–603.
7. Phillips, M. C. 2013. New insights into the determination of HDL structure by apolipoproteins: Thematic review series: high density lipoprotein structure, function, and metabolism. *J. Lipid Res.* **54:** 2034–2048.
8. Cheung, M. C., and J. J. Albers. 1982. Distribution of high density lipoprotein particles with different apoprotein composition: particles with A-I and A-II particles with A-I but not A-II. *J. Lipid Res.* **23:** 747–753.
9. Li, W-H., M. Tanimura, C-C. Luo, S. Datt, and L. Chan. 1988. The apolipoprotein multigene family: biosynthesis, structure, structure-function relationships, and evolution. *J. Lipid Res.* **29:** 245–271.
10. Ikewaki, K., L. A. Zech, H. B. Brewer, Jr., and D. J. Rader. 1996. ApoA-II kinetics in humans using endogenous labeling with stable isotopes: slower turnover of apoA-II compared with the exogenous radiotracer method. *J. Lipid Res.* **37:** 399–407.
11. Brewer, H. B. J., S. E. Lux, R. Ronan, and K. M. John. 1972. Amino acid sequence of human apoLp-Gln II (apoA-II), an apolipoprotein from the high density lipoprotein complex. *Proc. Natl. Acad. Sci. USA.* **69:** 1304–1308.
12. Lyssenko, N. N., M. Nickel, C. Tang, and M. C. Phillips. 2013. Factors controlling nascent high-density lipoprotein particle heterogeneity: ATP-binding cassette transporter A1 activity and cell lipid and apolipoprotein AI availability. *FASEB J.* **27:** 2880–2892.
13. Rothblat, G. H., and M. C. Phillips. 2010. High-density lipoprotein heterogeneity and function in reverse cholesterol transport. *Curr. Opin. Lipidol.* **21:** 229–238.
14. de Beer, M. C., L. W. Castellani, L. Cai, A. J. Stromberg, F. C. de Beer, and D. R. van der Westhuyzen. 2004. ApoA-II modulates the association of HDL with class B scavenger receptors SR-BI and CD36. *J. Lipid Res.* **45:** 706–715.
15. Lagrost, L., L. Perségol, C. Lallemant, and P. Gambert. 1994. Influence of apolipoprotein composition of high density lipoprotein particles on cholesteryl ester transfer protein activity. Particles containing various proportions of apolipoproteins AI and AII. *J. Biol. Chem.* **269:** 3189–3197.
16. Marzal-Casacuberta, A., F. Blanco-Vaca, B. Y. Ishida, J. Julve-Gil, J. Shen, S. Calvet-Márquez, F. Conzález-Sastre, and L. Chan. 1996. Functional lecithin:cholesterol acyltransferase deficiency and high density lipoprotein deficiency in transgenic mice overexpressing human apolipoprotein A-II. *J. Biol. Chem.* **271:** 6720–6728.
17. von Hodenberg, E., S. Heinen, K. E. Howell, C. Luley, W. Kubler, and H. M. Bond. 1991. Cholesterol efflux from macrophages mediated by high-density lipoprotein subfractions, which differ principally in apolipoprotein A-I and apolipoprotein A-II ratios. *Biochim. Biophys. Acta.* **1086:** 173–184.
18. Johnson, W. J., E. P. C. Kilsdonk, A. van Tol, M. C. Phillips, and G. H. Rothblat. 1991. Cholesterol efflux from cells to immunopurified

subfractions of human high density lipoprotein: LP-AI and LP-AI/AII. *J. Lipid Res.* **32:** 1993–2000.
19. Timmins, J. M., J. Y. Lee, E. Boudyguina, K. D. Kluckman, L. R. Brunham, A. Mulya, A. K. Gebre, J. M. Coutinho, P. L. Colvin, T. L. Smith, et al. 2005. Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J. Clin. Invest.* **115:** 1333–1342.
20. Liu, L., A. E. Bortnick, M. Nickel, P. Dhanasekaran, P. V. Subbaiah, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 2003. Effects of apolipoprotein A-I on ATP-binding cassette transporter A1-mediated efflux of macrophage phospholipid and cholesterol: formation of nascent high density lipoprotein particles. *J. Biol. Chem.* **278:** 42976–42984.
21. Weisweiler, P. 1987. Isolation and quantitation of apolipoproteins A-I and A-II from human high-density lipoproteins by fast-protein liquid chromatography. *Clin. Chim. Acta.* **169:** 249–254.
22. Gillotte, K. L., M. Zaiou, S. Lund-Katz, G. M. Anantharamaiah, P. Holvoet, A. Dhoest, M. N. Palgunachari, J. P. Segrest, K. H. Weisgraber, G. H. Rothblat, et al. 1999. Apolipoprotein-mediated plasma membrane microsolubilization - Role of lipid affinity and membrane penetration in the efflux of cellular cholesterol and phospholipid. *J. Biol. Chem.* **274:** 2021–2028.
23. Duong, P. T., H. L. Collins, M. Nickel, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 2006. Characterization of nascent HDL particles and microparticles formed by ABCA1-mediated efflux of cellular lipids to apoA-I. *J. Lipid Res.* **47:** 832–843.
24. Chetty, P. S., D. Nguyen, M. Nickel, S. Lund-Katz, L. Mayne, S. W. Englander, and M. C. Phillips. 2013. Comparison of apoA-I helical structure and stability in discoidal and spherical HDL particles by HX and mass spectrometry. *J. Lipid Res.* **54:** 1589–1597.
25. Rosales, C., B. K. Gillard, H. S. Courtney, F. Blanco-Vaca, and H. J. Pownall. 2009. Apolipoprotein modulation of streptococcal serum opacity factor activity against human plasma high-density lipoproteins. *Biochemistry.* **48:** 8070–8076.
26. Huang, R., R. A. Silva, W. G. Jerome, A. Kontush, M. J. Chapman, L. K. Curtiss, T. J. Hodges, and W. S. Davidson. 2011. Apolipoprotein A-I structural organization in high-density lipoproteins isolated from human plasma. *Nat. Struct. Mol. Biol.* **18:** 416–422.
27. Thrift, R. N., T. M. Forte, B. E. Cahoon, and V. G. Shore. 1986. Characterization of lipoproteins produced by the human liver cell line, Hep G2, under defined conditions. *J. Lipid Res.* **27:** 236–250.
28. Cheung, M. C., K. D. Lum, C. G. Brouillette, and C. L. Bisgaier. 1989. Characterization of apoA-I-containing lipoprotein subpopulations secreted by HepG2 cells. *J. Lipid Res.* **30:** 1429–1436.
29. Gillard, B. K., H. Y. Lin, J. B. Massey, and H. J. Pownall. 2009. Apolipoproteins A-I, A-II and E are independently distributed among intracellular and newly secreted HDL of human hepatoma cells. *Biochim. Biophys. Acta.* **1791:** 1125–1132.
30. McCall, M. R., T. M. Forte, and V. G. Shore. 1988. Heterogeneity of nascent high density lipoproteins secreted by the hepatoma-derived cell line, Hep G2. *J. Lipid Res.* **29:** 1127–1137.
31. Vedhachalam, C., L. Liu, M. Nickel, P. Dhanasekaran, G. M. Anantharamaiah, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 2004. Influence of apoA-I structure on the ABCA1-mediated efflux of cellular lipids. *J. Biol. Chem.* **279:** 49931–49939.
32. Remaley, A. T., J. A. Stonik, S. J. Demosky, E. B. Neufeld, A. V. Bocharov, T. G. Vishnyakova, T. L. Eggerman, A. P. Patterson, N. J. Duverger, S. Santamarina-Fojo, et al. 2001. Apolipoprotein specificity for lipid efflux by the human ABCAI transporter. *Biochem. Biophys. Res. Commun.* **280:** 818–823.
33. Krimbou, L., H. Hajj Hassan, S. Blain, S. Rashid, M. Denis, M. Marcil, and J. Genest. 2005. Biogenesis and speciation of nascent apoA-I-containing particles in various cell lines. *J. Lipid Res.* **46:** 1668–1677.
34. Schultz, J. R., E. L. Gong, M. R. McCall, A. V. Nichols, S. M. Clift, and E. M. Rubin. 1992. Expression of human apolipoprotein A-II and its effect on high density lipoproteins in transgenic mice. *J. Biol. Chem.* **267:** 21630–21636.
35. Lund-Katz, S., N. N. Lyssenko, M. Nickel, D. Nguyen, P. Sevugan Chetty, G. Weibel, and M. C. Phillips. 2013. Mechanisms responsible for the compositional heterogeneity of nascent high density lipoprotein. *J. Biol. Chem.* **288:** 23150–23160.
36. Edelstein, C., M. Halari, and A. M. Scanu. 1982. On the mechanism of the displacement of apolipoprotein A-I by apolipoprotein A-II from the high density lipoprotein surface. Effect of concentration and molecular forms of apolipoprotein A-II. *J. Biol. Chem.* **257:** 7189–7195.
37. Ibdah, J. A., K. E. Krebs, and M. C. Phillips. 1989. The surface properties of apolipoproteins A-I and A-II at the lipid/water interface. *Biochim. Biophys. Acta.* **1004:** 300–308.
38. van Tornout, P., R. Vercaemst, M. J. Lievens, H. Caster, M. Rosseneu, and G. Assmann. 1980. Reassembly of human apoproteins A-I and A-II with unilamellar phosphatidylcholine-cholesterol liposomes. Association kinetics and characterization of the complexes. *Biochim. Biophys. Acta.* **601:** 509–523.
39. Silva, R. A., L. A. Schneeweis, S. C. Krishnan, X. Zhang, P. H. Axelsen, and W. S. Davidson. 2007. The structure of apolipoprotein A-II in discoidal high density lipoproteins. *J. Biol. Chem.* **282:** 9713–9721.
40. Segrest, J. P., M. C. Cheung, and M. K. Jones. 2013. Volumetric determination of apolipoprotein stoichiometry of circulating HDL subspecies. *J. Lipid Res.* **54:** 2733–2744.
41. Vedhachalam, C., A. B. Ghering, W. S. Davidson, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 2007. ABCA1-induced cell surface binding sites for ApoA-I. *Arterioscler. Thromb. Vasc. Biol.* **27:** 1603–1609.
42. Vedhachalam, C., P. T. Duong, M. Nickel, D. Nguyen, P. Dhanasekaran, H. Saito, G. H. Rothblat, S. Lund-Katz, and M. C. Phillips. 2007. Mechanism of ATP-binding cassette transporter A1-mediated cellular lipid efflux to apolipoprotein A-I and formation of high density lipoprotein particles. *J. Biol. Chem.* **282:** 25123–25130.
43. Clay, M. A., D. H. Pyle, K. A. Rye, and P. J. Barter. 2000. Formation of spherical, reconstituted high density lipoproteins containing both apolipoproteins A-I and A-II is mediated by lecithin:cholesterol acyltransferase. *J. Biol. Chem.* **275:** 9019–9025.
44. Chisholm, J. W., E. R. Burleson, G. S. Shelness, and J. S. Parks. 2002. ApoA-I secretion from HepG2 cells: evidence for the secretion of both lipid-poor apoA-I and intracellularly assembled nascent HDL. *J. Lipid Res.* **43:** 36–44.
45. Sahoo, D., T. C. Trischuk, T. Chan, V. A. Drover, S. Ho, G. Chimini, L. B. Agellon, R. Agnihotri, G. A. Francis, and R. Lehner. 2004. ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes. *J. Lipid Res.* **45:** 1122–1131.
46. Kiss, R. S., D. C. McManus, V. Franklin, W. L. Tan, A. McKenzie, G. Chimini, and Y. L. Marcel. 2003. The lipidation by hepatocytes of human apolipoprotein A-I occurs by both ABCA1-dependent and -independent pathways. *J. Biol. Chem.* **278:** 10119–10127.
47. Tsujita, M., C. A. Wu, S. Abe-Dohmae, S. Usui, M. Okazaki, and S. Yokoyama. 2005. On the hepatic mechanism of HDL assembly by the ABCA1/apoA-I pathway. *J. Lipid Res.* **46:** 154–162.
48. Gordon, J. I., H. F. Sims, S. R. Lentz, C. Edelstein, A. M. Scanu, and A. W. Strauss. 1983. Proteolytic processing of human preproapolipoprotein A-I. A proposed defect in the conversion of pro A-I to A-I in Tangier's disease. *J. Biol. Chem.* **258:** 4037–4044.
49. Gordon, J. I., K. A. Budelier, H. F. Sims, C. Edelstein, A. M. Scanu, and A. W. Strauss. 1983. Biosynthesis of human preproapolipoprotein A-II. *J. Biol. Chem.* **258:** 14054–14059.
50. Remaley, A. T., A. W. Wong, U. K. Schumacher, M. S. Meng, H. B. Brewer, Jr., and J. M. Hoeg. 1993. *O*-Linked glycosylation modifies the association of apolipoprotein A-II to high density lipoproteins. *J. Biol. Chem.* **268:** 6785–6790.
51. Maric, J., R. S. Kiss, V. Franklin, and Y. L. Marcel. 2005. Intracellular lipidation of newly synthesized apolipoprotein A-I in primary murine hepatocytes. *J. Biol. Chem.* **280:** 39942–39949.
52. Zheng, H., R. S. Kiss, V. Franklin, M. D. Wang, B. Haidar, and Y. L. Marcel. 2005. ApoA-I lipidation in primary mouse hepatocytes. Separate controls for phospholipid and cholesterol transfers. *J. Biol. Chem.* **280:** 21612–21621.
53. Durbin, D. M., and A. Jonas. 1997. The effect of apolipoprotein A-II on the structure and function of apolipoprotein A-I in a homogeneous reconstituted high density lipoprotein particle. *J. Biol. Chem.* **272:** 31333–31339.
54. Gauthamadasa, K., N. S. Vaitinadin, J. L. Dressman, S. Macha, R. Homan, K. D. Greis, and R. A. Silva. 2012. Apolipoprotein A-II-mediated conformational changes of apolipoprotein A-I in discoidal high density lipoproteins. *J. Biol. Chem.* **287:** 7615–7625.
55. Gao, X., S. Yuan, S. Jayaraman, and O. Gursky. 2012. Role of apolipoprotein A-II in the structure and remodeling of human high-density lipoprotein (HDL): protein conformational ensemble on HDL. *Biochemistry.* **51:** 4633–4641.