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Original Research

ApoA-I Dissociated From Human HDL Retains its Acceptor Properties in ABCA1-mediated Cholesterol Efflux From RAW 264.7 Macrophages in Coronary Artery Disease

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Abstract

Background: The significance of cholesterol efflux as a predictor of coronary artery disease (CAD) remains controversial. The intracellular cholesterol export via the ABCA1 transporter involves the acceptance of cholesterol by both lipid-free apolipoprotein A-I and high-density lipoproteins (HDL). An estimate of the efficiencies of two reactions is thus required. **Methods**: HDL from the plasma of 63 control and 76 male CAD patients was obtained by the precipitation of apoB-containing lipoproteins and denatured by urea. We measured apoA-I dissociation concomitant with HDL denaturation by agarose gel electrophoresis followed by immunodetection and the expression of 65 preselected genes in blood mononuclear cells by real-time PCR. The total cholesterol efflux capacity (CEC) of ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux from RAW 264.7 macrophages, when preβ-HDL and α-HDL act as competitive inhibitors of each other for the binding to ABCA1 transporter, was measured with intact HDL and pre-denatured HDL as a source of lipid-free apoA-I. **Results**: The phospholipid:apoA-I and cholesterol:apoA-I ratios in HDL from CAD patients were higher than those for control patients across the full range of plasma HDL-cholesterol levels. ApoA-I partitioned 1.5-fold higher into the water phase for HDL from CAD patients relative to controls. In CAD patients, the dissociation parameter *D* was inversely correlated with absolute and normalized per apoA-I phospholipid and cholesterol levels in HDL. For control patients, the *D* parameter was positively correlated with *ABCA1* gene expression. For CAD patients, the *D* parameter was positively correlated with *PLTP* and inversely with *CUBN* and *ALB* gene expression. ApoA-I functionality in ABCA1-mediated cholesterol efflux from RAW 264.7 macrophages to lipid-free apoA-I generated from urea-induced HDL denaturation was similar for HDL from control and CAD groups. The retained CEC of lipid-free apoA-I in CAD may be masked by competition with α-HDL, which has a lower CEC, for ABCA1 binding to preβ-HDL. **Conclusions**: The enrichment of HDL with cholesterol and phospholipids may contribute to the increased apoA-I dissociation from HDL in CAD. Estimates of both lipid-free apoA-I and intact HDL may be a prerequisites for a detailed study of ABCA1-mediated cholesterol efflux, which could allow these apoA-I forms to be identified as CAD predictors.

Keywords: ABCA1; apolipoprotein A; cholesterol; coronary artery disease; gene expression; high-density lipoprotein; lipid-bound; lipid-free

1. Introduction

Human plasma high-density lipoproteins (HDL) possess an atheroprotective effect through the reverse cholesterol transport from arterial-wall macrophages to the liver [1], with cholesterol efflux as a first step. The efflux includes active cholesterol transport by ABCA1 and ABCG1 transporters, facilitated diffusion with SR-B1, and passive diffusion [2]. ApoA-I is a major HDL apolipoprotein that exists in lipid-bound and lipid-free forms. The lipid-free apoA-I is a primary cholesterol acceptor in the ABCA1-mediated efflux. However, small HDL particles are efficient cholesterol acceptors as well [3]. The HDL heterogeneity by density and charge results in the existence of light HDL_2 and dense HDL_3 and particles with preβ-mobility and α-mobility, respectively. Lipid-free/lipid-poor apoA-I (preβ_1-apoA-I) is found only in the preβ-band, which also includes nascent HDL (preβ_2-apoA-I), while mature spherical HDL is localized in the α-band. Lipid-free and lipid-poor apoA-I terms are used interchangeably throughout the text. The nascent HDL, as the initial product of cholesterol and phospholipid efflux by ABCA1, further accepts these lipids with the formation of disc-shaped particles. These particles are the most efficient substrate for lecithin:cholesterol acyltransferase (LCAT). The enzyme converts free cholesterol to cholesteryl ester that is thought to prevent the reabsorption of effluxed cholesterol. The subsequent steps of reverse cholesterol transport include the transfer of cholesteryl ester from HDL to larger very low-density (VLDL) and low-density lipoproteins (LDL) by cholesteryl ester transfer protein (CETP), the SR-B1-mediated capture of cholesteryl ester, and the uptake of a whole LDL particle by the LDL receptor in the liver

[2]. The remodeling of spherical HDL by CETP and lipases results in a spherical-to-discoidal transformation with a concomitant transition between lipid-free and lipid-bound apoA-I [4]. PLTP is a putative fusion factor to make bigger HDL with concomitant apoA-I release [5]. Of note, the dissociated apoA-I [6,7] and HDL-apoA-I exchange [8] may be potentially involved in cholesterol efflux.

A strong inverse relationship between HDL cholesterol efflux capacity (CEC) and coronary artery disease (CAD) signifies HDL functionality more than HDL-cholesterol level in the HDL atheroprotective effect [9]. HDL functionality includes HDL particle number and heterogeneity and particle ability to accept effluxed cholesterol. The significance of ABCA1-mediated efflux and HDL functionality in CAD are inconsistent [3,10–15]. CEC from macrophages had a strong inverse association with morphological and prognostic markers of angiographic CAD [16]. The higher concentration of preβ_1 particles was responsible for the lower preβ_1 concentration-normalized ABCA1-dependent efflux capacity in CAD [17,18]. ABCA1-dependent cholesterol efflux was positively correlated with the levels of small lipid-poor preβ_1 particles [17]. At variance with this, HDL efflux capacity was inversely associated with small HDL particle concentration in older adults [19]. Furthermore, the association between HDL particle size and composition and CAD risk is not conclusive, and the role of lipid-free apoA-I and HDL particles in individual steps of cholesterol efflux remains to be implicitly solved as well.

The major goals of our study were to quantitatively follow urea-induced denaturation of HDL from control and CAD patients by apoA-I dissociation and to use generated lipid-free apoA-I to measure the efficacy of cholesterol efflux by a single ABCA1 transporter to two competing substrates—lipid-free apoA-I and mature HDL. We describe also the relations between apoA-I dissociation and the expression of selected genes controlling HDL metabolism and atherogenesis in control and CAD patients.

2. Materials and Methods

2.1 Patient Selection and HDL Isolation

Patient selection with widely varied HDL-C levels (63 control patients and 76 patients with CAD confirmed by coronary angiography) without lipid-lowering therapy, anthropometric data, and laboratory tests was described previously [20]. Only male patients were included in control and CAD groups. The crude HDL preparations (pHDL) were prepared by the precipitation of apoB-containing lipoproteins with PEG 7000–9000 [6]. The concentrations of choline-containing phospholipids and total cholesterol in HDL preparations were measured by enzyme methods using Sentinel CH. SpA (lot 90398; ref: 17320, Milano, Italy) and HUMAN GmbH (lot 23005, ref 10028, Wiesbaden, Germany) kits, respectively, and apoA-I was measured by immunonephelometry with AU 480 (Beckman Coulter Inc, Brea, CA, USA).

2.2 pHDL Charge Heterogeneity

ApoA-I content in preβ- and α-fractions of pHDL was measured by agarose gel electrophoresis followed by immunodetection of apoA-I [6]. PeakFit version 4.12 (SeaSolve Software Inc. Framingham, MA, USA) and ImageJ version 1.54h (https://wsr.imagej.net/ij/index.html) software were used to determine band intensities with baseline subtraction at the conditions of linear response of each band intensity to the amount of loaded sample.

2.3 Urea-induced ApoA-I Dissociation

Urea-induced pHDL denaturation after incubation for 6 h at 25 °C was followed by the accumulation of lipid-free preβ-apoA-I detected in the immunoreplica of agarose gel. ApoA-I dissociation was analyzed with the fractions of preβ-signal detected after the treatment with 0 M, 4.25 M, and 7.5 M urea [6]. The degree of apolipoprotein dissociation at 4.25 M urea as a mid-transition zone that is accomplished at 7.5 M urea was characterized by dissociation parameter *D* (Eqn. 1):

$$\mathrm{D} = (\mathrm{pre}\,\beta'_{4.25} - \mathrm{pre}\,\beta'_{0}) \,/\, (\mathrm{pre}\,\beta'_{7.5} - \mathrm{pre}\,\beta'_{0}) \quad (1)$$

where $pre\beta'_{4.25}$, $pre\beta'_{0}$, and $pre\beta'_{7.5}$ are preβ fractions with 4.25 M, 0 M, and 7.5 M urea, respectively. The dependence of urea-induced apoA-I release on phospholipid level in pHDL preparations was characterized by the partition coefficient *K* between aqueous and lipid phases adjusted for water molarity (Eqn. 2):

$$K = (F/B)[\mathrm{pHDL} - \mathrm{PL}] \quad (2)$$

where *F* and *B* are the numbers of molecules of lipid-free apoA-I in water and bound to pHDL phospholipids, respectively [21]. The *F*/*B* ratio was calculated from the *D* parameter measured at 4.25 M urea (Eqn. 3):

$$F/B = D/(1 - D) \quad (3)$$

2.4 Cholesterol Efflux Measurements

Cholesterol efflux was measured with the murine macrophage cell line RAW 264.7, which is a standard cell culture for the measurement of ABCA1-mediated cholesterol efflux [15,22]. The cell line was obtained from the European Collection of Cell Cultures and validated by speciation by DNA-bar sequencing of the *COX* subunit 1 gene and tested negative for mycoplasma. Efflux from cells to pHDL as a cholesterol acceptor was measured with a fluorescent probe, BODIPY-cholesterol. The basal and cAMP-

stimulated effluxes were measured, and ABCA1-mediated efflux was calculated as the difference between stimulated and basal effluxes and expressed as a percent of effluxed cholesterol [6].

To separate the contributions of both preβ-HDL and mature α-HDL in ABCA1-mediated efflux, a simple kinetic theorem for a two-substrate reaction, when substrates react with a single enzyme without the formation of any ternary complex [23], was applied for a total value of ABCA1-mediated efflux with pHDL as a cholesterol acceptor. The total initial rate of reaction v_{tot} when preβ-HDL and α-HDL act as competitive inhibitors of each other for the binding to the ABCA1 transporter is described by Eqn. 4:

$$v_{\text{tot}} = \frac{\frac{V_1}{K_1}[S_1] + \frac{V_2}{K_2}[S_2]}{1 + \frac{[S_1]}{K_1} + \frac{[S_2]}{K_2}} \quad (4)$$

where V_i, K_i, and $[S_i]$ are the maximal velocities, Michaelis constants and substrate concentrations for preβ-HDL and α-HDL as first and second substrates, respectively, with the relation $V_1 > V_2$. Both $[S_1]$ and $[S_2]$ are apoA-I concentrations in the preβ-band and α-band. The treatment of Eqn. 4 in the double reciprocal plots for a first substrate at a constant $[S_2]/[S_1]$ ratio R results in Eqn. 5:

$$\frac{1}{v_{tot}} = \frac{\frac{1}{[S_1]} + \frac{1}{K_1} + \frac{R}{K_2}}{\frac{V_1}{K_1} + \frac{RV_2}{K_2}} \quad (5)$$

For a series of lines in double reciprocal plots for a first substrate with various R values, the common intersection point does not depend on R, and abscissa and ordinate values are determined by Eqns. 6,7, respectively:

$$\frac{1}{[S_1]} = \frac{V_1 - V_2}{K_1 V_2} \quad (6)$$

$$\frac{1}{V} = \frac{1}{V_2} \quad (7)$$

A common maximal velocity V_{m} at the constant R is determined by Eqn. 8:

$$V_m = \frac{V_1 K_2 + R V_2 K_1}{K_2 + R K_1} \quad (8)$$

In turn, the K_2 value is derived from Eqn. 8 (Eqn. 9):

$$K_2 = \frac{R K_1 (V_m - V_2)}{V_1 - V_m} \quad (9)$$

V_{m} varies within low V_2 and high V_1 borders. Generally, V_{m} non-linearly depends on R. The linear plots according to Eqn. 5 were constructed for fully denatured and intact pHDL sets, and the data were subsequently treated with Eqns. 6,7,8,9 to derive kinetic parameters. The V_1 and K_1 values were measured without S_2 interference with pHDL fully denatured with 7.5 M urea. Before mixing, predenatured HDL was diluted 1:100 to lower urea concentration. The residual urea concentration not exceeding 75 mM did not influence efflux.

2.5 Gene Expression

The expression of 65 genes involved in HDL metabolism and atherogenesis measured by us earlier by real-time PCR in peripheral blood mononuclear cells [20, 24] was compared to apoA-I dissociation from pHDL in the present study. Housekeeping genes included Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Lactate dehydrogenase A (*LDHA*), and Ribosomal protein L3 (*RPL3*) genes. The relative expression of all target genes was normalized by the geometric mean of three reference genes.

2.6 Statistical Analysis

Statistica version 13 (TIBCO Software Inc. (http://tibco.com)) software was used for statistical analysis. The associations between variables were analyzed by the Spearman rank order correlation coefficient. The statistical significance limit was accepted as $p < 0.05$. Nonlinear curve fitting was done with OriginPro 9.0.0 SR1 (OriginLab Corporation, MA, USA). The significance of the difference between different dataset fits was checked with the F-statistic.

3. Results

3.1 Relations Between ApoA-I Dissociation, HDL Composition, and Gene Expression

The dissociation of apoA-I from pHDL induced by urea treatment and measured by the D parameter significantly decreased with the increase of absolute and apoA-I-normalized cholesterol and phospholipid (PL) levels in pHDL from CAD patients (Table 1). The fit of dissociation data, passed through zero, to the linear model of apoA-I distribution between lipid-free (F) and lipid-bound (B) states (Eqn. 2) results in the values of the partition coefficient between aqueous and lipid phases adjusted for water molarity $K = 0.239 \pm 0.012$ ($R^2 = 0.874$) for control pHDL and $K = 0.368 \pm 0.020$ ($R^2 = 0.822$) for pHDL from CAD patients (Fig. 1). Thus, a 1.54-fold prevalence of apoA-I distribution between water and lipid phases exists in CAD relative to controls ($p = 0.000$) with the value of the difference in the standard free energy $\Delta\Delta G^0 = \Delta G^0_{CAD} - \Delta G^0_{control} =$ –RTln(K(CAD)/K(control)) = –1.2 kJ/mol; the difference value is positive for apolipoprotein distribution between lipid and water phases.

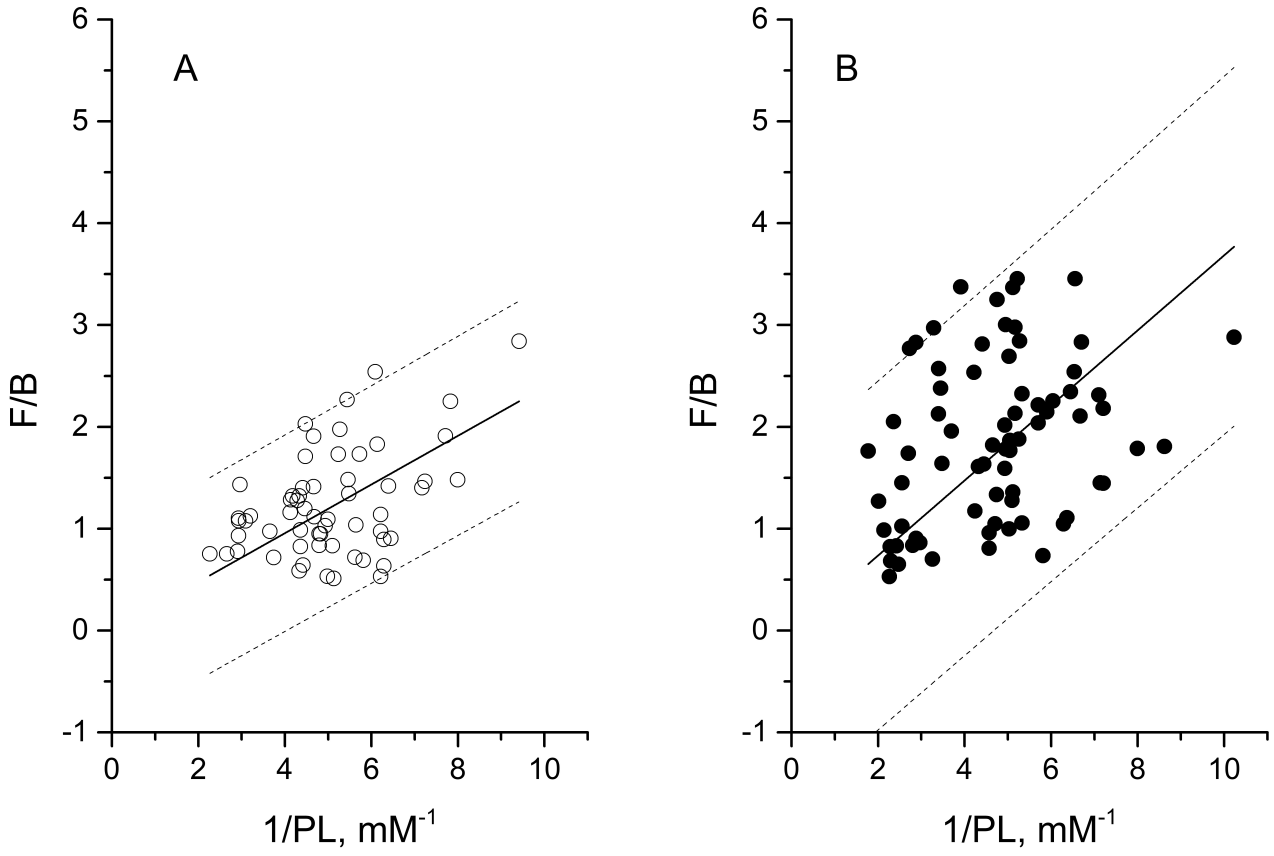

Fig. 1. ApoA-I distribution between water and lipid phases of pHDL predenatured by 4.25 M urea. (A) pHDL from control patients (n = 59). (B) pHDL from CAD patients (n = 73). The 95% prediction bands are given as thin dashed lines. The Y-intercepts passed through zero according to the distribution model. Slopes were 0.239 ± 0.012 (R^2 = 0.874) for control and 0.368 ± 0.020 (R^2 = 0.822) for CAD. Slope differences were significant with p = 0.000.

The similar values for free energy change have been obtained for the exchange of apoA-I variants between discoidal HDL-bound and lipid-free pools (1–3 kJ/mol) [25] and the lipid transfer for nanodisks consisting of apoA-I and DMPC (1.7 kJ/mol) [26]. ApoA-I in HDL from CAD patients possesses higher free energy and, thus, exhibits a higher dissociation. Of note, the increased PL:apoA-I and Chol:apoA-I ratios for pHDL from CAD patients relative to controls were measured throughout the whole range of HDL-C concentration (data not shown). To reveal the associations between apoA-I dissociation and lipoprotein metabolism, the expression of 65 genes in peripheral blood mononuclear cells, sensitive to HDL metabolism and atherogenesis [20,24], was analyzed. The significant correlations between D and gene expression are included in Table 1. For control patients, the positive correlations of the D parameter with the *ABCA1* and *TLR8* expression levels were revealed. For patients with CAD, the positive (for *PRKACB*, *HMGCR*, *PLTP*, *CD36*, *SLPI*, and *OLR1*) and inverse (for *CUBN*, *ALB*, and *IL1R1*) associations of gene expression in peripheral blood with the degree of apoA-I dissociation were revealed.

3.2 Relations Between ApoA-I and Cholesterol Efflux

First, the efflux measurements were done with intact pHDL as cholesterol acceptors. Both for control and CAD patients, there were significant inverse correlations between apoA-I dissociation and ABCA1-mediated cholesterol efflux (Table 1).

Second, due to the involvement of lipid-free and lipid-bound apoA-I in cholesterol transport by the ABCA1 transporter, the decrease of efflux efficiency in CAD may be associated with the less efficient efflux to each or both acceptors. To separate individual contributions, the functionality of lipid-free apoA-I was studied (Fig. 2). To induce apoA-I dissociation from the HDL lipid phase to the water phase, pHDL from control and CAD patients were denatured for 6 h at 25 °C by 4.25 M urea at the middle of the denaturation transition. The ABCA1-mediated efflux for 2 h to pHDL, at fixed or serial dilutions, was then measured, and the data were fitted to Michaelis-Menten kinetics. The kinetic parameters are given in Table 2. The concentration of lipid-free preβ-apoA-I dissociated from α-HDL was calculated as a product of dissociation parameter D and apoA-I level in the α-band estimated by agarose gel electrophoresis

Table 1. Spearman rank order correlation coefficients for correlation of dissociation parameter *D* with pHDL lipids, gene expression, and ABCA1-mediated cholesterol efflux.

Variable	Control	CAD
pHDL level		
pHDL-C	–0.256 (0.043)	–0.366 (0.001)
pHDL-PL	n.s.	–0.336 (0.003)
pHDL-PL:apoA-I	n.s.	–0.329 (0.004)
pHDL-C:apoA-I	n.s.	–0.344 (0.002)
Gene expression		
ABCA1	0.278 (0.029)	n.s.
APOA1	–0.357 (0.005)	n.s.
TLR8	0.352 (0.005)	n.s.
PRKACB	n.s.	0.250 (0.031)
CUBN	n.s.	–0.305 (0.008)
HMGCR	n.s.	0.274 (0.017)
PLTP	n.s.	0.242 (0.036)
ALB	n.s.	–0.280 (0.015)
CD36	n.s.	0.259 (0.025)
SLPI	n.s.	0.294 (0.011)
OLR1	n.s.	0.328 (0.004)
IL1R1	n.s.	–0.260 (0.024)
HDL function		
Chol efflux	–0.305 (0.015)	–0.300 (0.009)

Spearman rank order correlation coefficients *R* with significance level (*p*) are given for control (n = 63) and CAD (n = 75) patients. n.s. – nonsignificant. CAD, coronary artery disease; HDL, high-density lipoproteins; pHDL, HDL preparations.

and apolipoprotein immunodetection ($D \times \alpha$-apoA-I). The dependencies were well fitted to the equation for one substrate reaction and did not differ by the F-test for control and CAD. The V_m, K_m, and V_m/K_m kinetic parameters did not differ also for pHDL from control and CAD patients by Student's *t*-test. Of note, the kinetic parameters, checked by the F-statistic, did not change significantly with pHDL predenatured with 7.5 M urea (Table 2) at the completion of the denaturation transition of pHDL from control or CAD patients, which assumes no contribution of HDL remaining at the transition midpoint to the cholesterol efflux. Thus, the lipid-free apoA-I seems to be equally efficient as an acceptor of cholesterol effluxed by ABCA1 in CAD relative to control samples. We used this approach to separate the contributions of lipid-free and HDL-bound apoA-I as first and second substrates, respectively, to the kinetics of ABCA1-mediated efflux based on the simple kinetic theorem of two substrates competing for one enzyme (Eqn. 4). The initial rates of efflux to intact and fully denatured pHDL from CAD patients with low HDL-C were treated as a function of preβ-apoA-I concentration [S_1] in double reciprocal plots (Fig. 3). The major prerequisite for a plot linearity is a constant [S_2]/[S_1] ratio *R* (Eqn. 5). The efflux was measured separately with two intact pHDLs with similar *R* values and five denatured pHDLs, and the data were concatenated to increase the statistical power. The measurements with denatured pHDL were necessary to derive V_1 and K_1 values without S_2 interference. The V_2 and K_2 values for intact pHDL with an *R* value 13.0 were derived with Eqns. 6,7,8,9. The complete set of mean values of kinetic parameters included V_1 = 17.5 %/2 h, K_1 = 0.62 µg/mL, V_2 = 5.3 %/2 h, and K_2 = 2.13 µg/mL. Thus, efflux efficiency, expressed as the V_m/K_m ratio, for lipid-free apoA-I was 11.4-fold higher than that for HDL-bound apoA-I in HDL from CAD patients with low HDL-C levels.

4. Discussion

4.1 Study Prerequisite

We used PEG 7000–9000 to prepare crude HDL for subsequent cholesterol efflux measurements. ApoB depletion by PEG is the most frequent approach in such studies [15,22,27] due to quick and efficient plasma/serum fractionation with preservation of HDL integrity. To avoid possible contamination by apoB-containing aggregated lipoproteins, we discarded the thin surface layer after the centrifugation step, if present. Importantly, the procedure, if performed at room temperature, neither involves apoA-I dissociation [28] nor an overall efflux change [29]. These properties are crucial in the preservation of individual efflux steps with lipid-free apoA-I and α-HDL-bound apoA-I. Also, HDL isolation by ultracentrifugation leads to apoA-I loss and alters HDL size distribution [22], while chromatography leads to sample dilution and the additional step of fraction concentration; both procedures are time-consuming. The knowledge of HDL heterogeneity by size or density could detail the origin of differences in apoA-I dissociation induced by urea; however, it preserves the major conclusion on the similar cholesterol-accepting properties of lipid-free apoA-I from CAD and control patients. We used murine macrophages RAW 264.7 as a standard cell line to test the cholesterol-accepting capacity of individual pHDL when ABCA1 properties were inwardly preserved. These measurements focus on the interaction between the cholesterol acceptor and the transporter and not on the effect of the intervention on ABCA1 expression [15]. We used the intact and c-AMP-treated cells to specifically induce ABCA1 expression in murine macrophages and included the control sample on the different plates to perform inter-plate standardization of ABCA1-mediated cholesterol efflux. BODIPY-cholesterol is also a more sensitive probe for ABCA1-mediated efflux than radiolabeled cholesterol [30].

4.2 Mechanistic Insight

There are three major findings in the present study revealed by the comparison of compositional and functional properties of HDL from CAD and control patients.

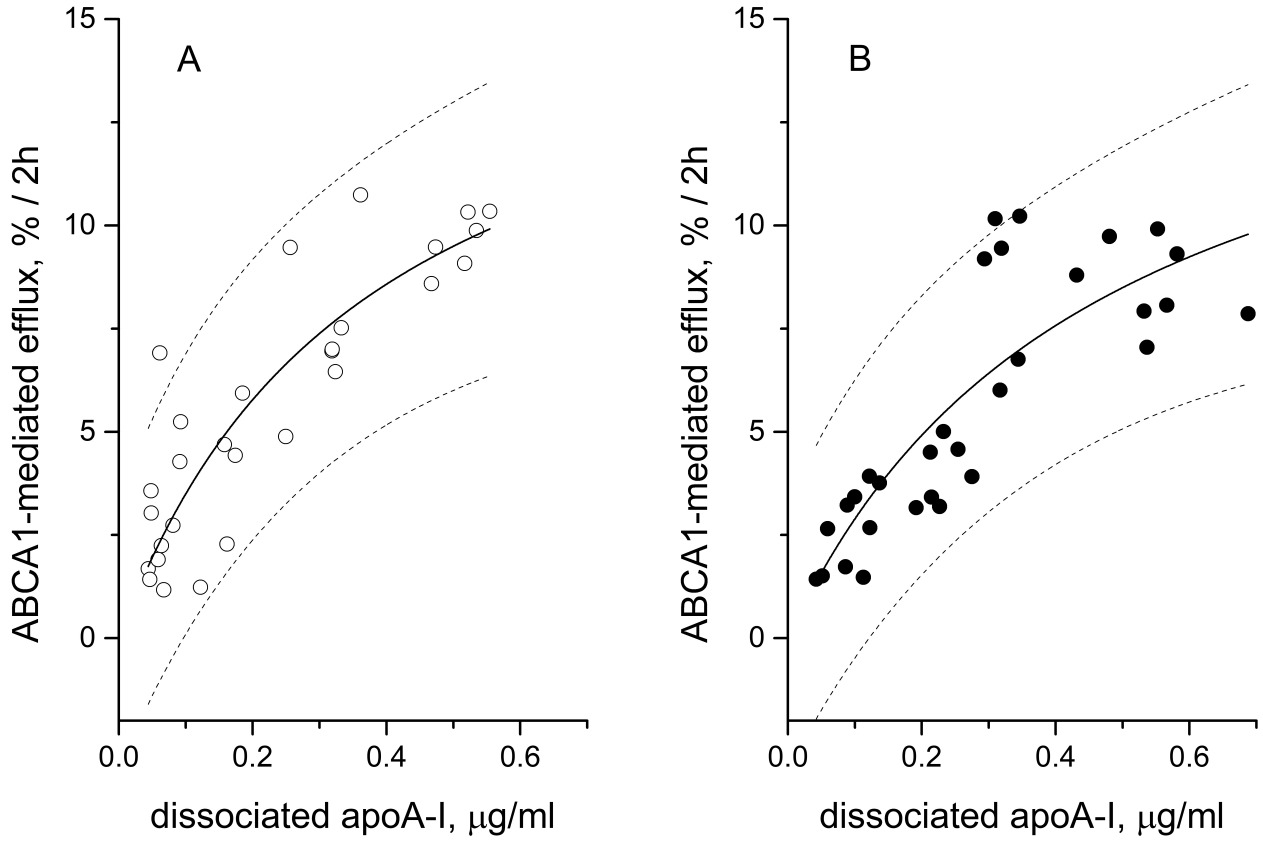

Fig. 2. The kinetics of ABCA1-mediated efflux to pHDL predenatured by 4.25 M urea. (A) pHDL from control patients (n = 29). (B) pHDL from CAD patients (n = 31). The 95% prediction bands are given as thin dashed lines.

First, for HDL from CAD patients, apoA-I partitioned more into the water phase during urea-induced HDL denaturation. ApoA-I dissociation is determined by the equilibrium [7,31] and/or kinetic [32] nature of HDL stability. The first condition implies apoA-I distribution between lipid and water phases by mass law action, while the second condition includes apolipoprotein shedding from the HDL surface induced by metabolic transformation. Metabolic remodeling that includes the transitions between different lipoprotein structures is accompanied by the apolipoprotein A-I dissociation from the HDL surface and particle fusion; lipolytic and transfer activities are the most important. CETP- and HL-induced dissociation of lipid-free apoA-I from HDL with the subsequent formation of discoidal HDL occurs in the presence of VLDL or LDL [33,34]. This apoA-I dissociation also occurs at HDL thermal or chemical denaturation [6,21,32,35]. The chaotropic agent-induced apoA-I dissociation is irreversible [21], and HDL denaturation induces apoA-I unfolding and partitioning from HDL to water (Fig. 1). Partitioning also occurred in the absence of denaturants [7] that may result in the appearance of lipid-free apoA-I as a principal ligand for the ABCA1 transporter. The spontaneous exchange of apoA-I between lipid-free and lipid-bound forms [8,36] differs from dissociation, as no net transfer occurs in the exchange. Irrespective of the nature of dissociation, this process results in the existence of up to 10% of total plasma apoA-I in lipid-free form.

Distribution parameter *D* was inversely associated with the absolute and apoA-I-normalized levels of choline-containing phospholipids and cholesterol in pHDL, opposite to the lack of any association for pHDL from control patients. The increased denaturation of HDL from patients with acute coronary syndrome has been described [35]. Importantly, HDL particles from CAD patients are more enriched with PL and cholesterol normalized by apoA-I level throughout the whole range of HDL-C. The enrichment of HDL with cholesterol, with the concomitant increase of competition between apoA-I and cholesterol for the binding to phospholipid molecules adjacent to apoA-I, is suggested to be involved in the increased apolipoprotein dissociation from HDL in CAD. Cholesterol molecules have been shown to be partially excluded from boundary lipids in reconstituted HDL [37]. The free cholesterol molecules may increasingly accumulate in the vicinity of apoA-I molecules in cholesterol-overloaded pHDL from CAD patients with the additional perturbation of PL structural order and dynamics. What could be a driving force for additional cholesterol accumulation in HDL from CAD patients compared to control patients? Earlier, we measured the upregulated expression of *CETP*, *LPL*, and *PLTP* genes and the downregu-

Table 2. Kinetics of ABCA1-mediated efflux to pHDL predenatured by urea.

Group	Urea, M	n	V_m, %/2 h	K_m, µg/mL	V_m/K_m
Control	4.25	29	16.6 ± 3.5	0.38 ± 0.15	44.2 ± 19.7
	4.25	5	16.8 ± 3.5	0.69 ± 0.27	24.4 ± 10.9
	7.50	5	15.0 ± 1.9	0.74 ± 0.22	20.3 ± 6.6
CAD	4.25	31	16.5 ± 3.9	0.47 ± 0.20	35.0 ± 16.9
	4.25	4	18.9 ± 1.6	0.61 ± 0.11	30.8 ± 6.0
	7.50	4	17.7 ± 1.0	0.62 ± 0.08	28.3 ± 4.1

Mean ± SE values are given. n, number of v_{tot} points with a fixed 1:100 dilution (29 and 31) or number of pHDL (5 and 4) with a varied dilution in efflux kinetics.

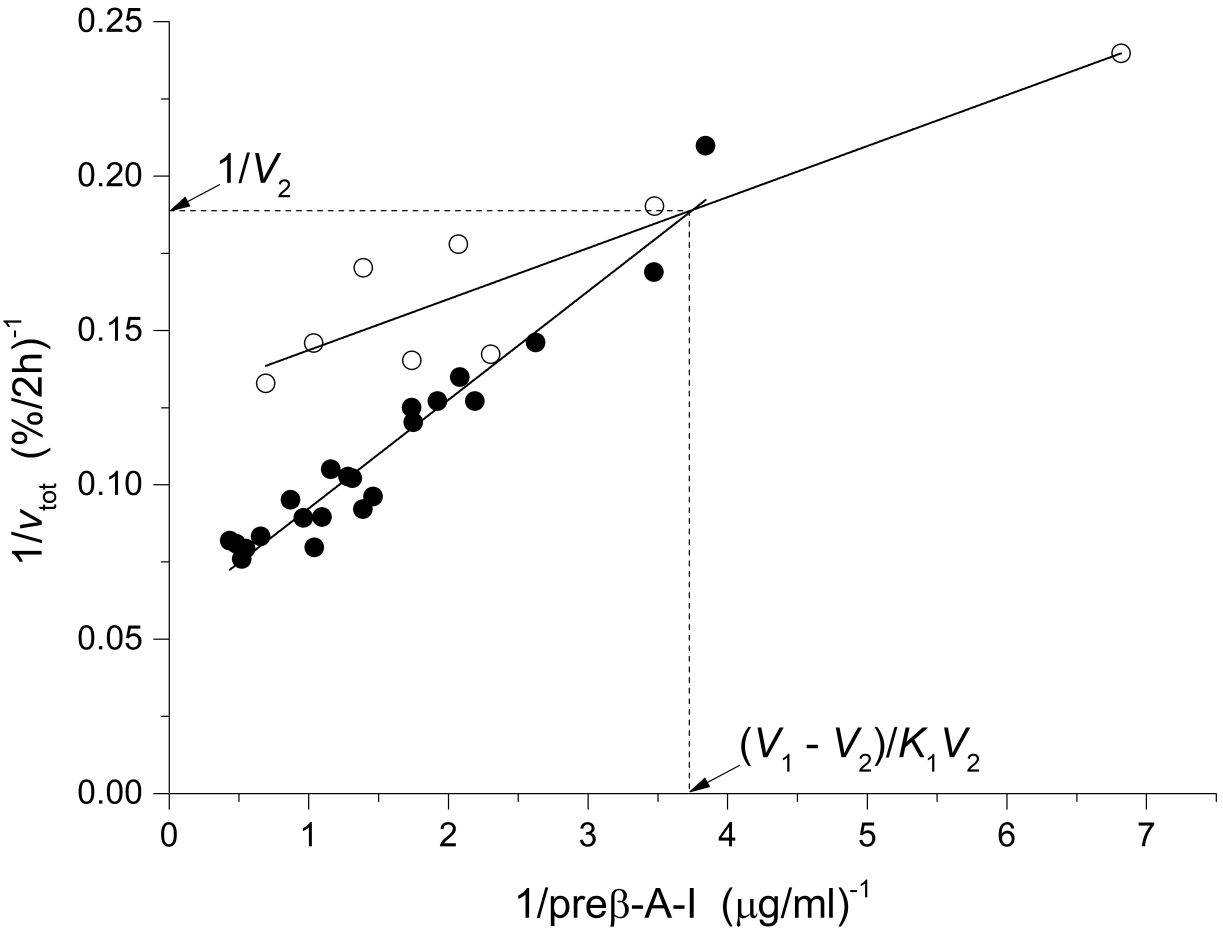

Fig. 3. The dependency of the total initial rates of ABCA1-mediated cholesterol efflux v_{tot} on S_1 concentration in double reciprocal plots for intact and denatured pHDL from the CAD group with low plasma HDL-C levels. The data were fitted to a linear function $1/v_{tot} = intercept + slope \times (1/[S_1])$. The efflux was measured with five individual pHDL pre-denatured with 7.5 M urea (filled symbols) and treated with concatenated data: the *intercept* was 0.0573 ± 0.003 ($p = 0.000$), the *slope* was 0.035 ± 0.002 ($p = 0.000$), and $R^2 = 0.94$. For two intact pHDL with a mean value of $R = 13.0$ (open symbols), the *intercept* was 0.127 ± 0.009 ($p = 0.000$), the *slope* was 0.017 ± 0.003 ($p = 0.002$), and $R^2 = 0.83$. The kinetic parameters with S_2 were calculated from the ordinate and abscissa of the intersection point.

lated expression of the *LCAT* gene among 65 genes selected to follow atherogenesis-prone changes in CAD [20,24]. The differential expression of these genes is suggested to be involved in lipid enrichment of HDL in CAD. Indeed, the enrichment of HDL with phospholipid and cholesterol molecules in CAD could occur due to a decreased phospholipid hydrolysis by LCAT, which possesses phospholipase activity with a concomitant lowering of cholesteryl ester generation. PLTP is capable of transforming small into large HDL particles with a concomitant release of apoA-I

[5]; the effect of upregulated expression of the *PLTP* gene lies in accordance with the increase of apoA-I-normalized cholesterol and phospholipid levels in CAD. The entropically favorable processes of both apoA-I exchange between discoidal HDL-bound and lipid-free pools [25] and the lipid transfer for nanodisks consisting of apoA-I and DMPC [26] with the increased activation entropy and the decrease in the standard entropy have been suggested. The exact contribution of entropy and enthalpy to apoA-I increased dissociation from mature spherical HDL in CAD remains to be determined. The different exposure of the hydrophobic amino acid residues of apoA-I to the aqueous phase in the activated state at apolipoprotein denaturation [21] may be involved. Interestingly, cholesterol in discoidal HDL increased the number of apoA-I tryptophan residues accessible to the aqueous phase but decreased their mean degree of hydration [38].

Second, we revealed significant associations between the apoA-I dissociation parameter and expression of several genes (Table 1). These associations may reveal proatherogenic or atheroprotective effects, and any causal links remain to be separately studied. For HDL from control patients, the *D* parameter was positively correlated with the *ABCA1* and *TLR8* expressions. The decrease of lipid-free apoA-I with the increase of ABCA1 activity is suggested to result in the increase of dissociation of lipid-bound apolipoprotein. Of note, the increased apoA-I dissociation seems to possess a proatherogenic effect in control patients due to the positive associations between the *D* parameter and *TLR8* gene expression, which is involved in inflammation [39].

For HDL from CAD patients, the D parameter was associated with the expression of a number of genes involved in lipoprotein metabolism (*PRKACB*, *HMGCR*, *PLTP*, *OLR1*, *CUBN*, and *ALB*) and systemic inflammation (*CD36, SLPI*, and *IL1R1*). Interestingly, the expression of apoptosis-related genes *IL1R1* and *PRKACB* with the inverse associations with the D parameter has also been regulated inversely in acute myocardial infarction [40]. The observed inverse associations of the *D* parameter with *CUBN* and *ALB* gene expression may be associated with the increased catabolism of lipid-free apoA-I and the accompanying decrease of the preβ fraction in CAD. Indeed, CUBN, as an endocytic receptor, is involved in endocytosis of apoA-I and albumin and maintenance of their blood levels [41]. Mechanistically, PLTP activity promotes both phospholipid transfer from VLDL and LDL to HDL and preβ_1-HDL formation by loss of apoA-I from larger HDL particles [42,43], thus explaining the positive association between *PLTP* gene expression and the *D* parameter in CAD. However, this association seems not to be significant for control patients due to the efficient transformation of preβ_1-HDL to larger HDL by LCAT activity in the preβ_1-HDL cycle [43], which is suggested to be impaired in CAD. For controls, normal PLTP activity with generation of preβ_1-HDL is balanced by normal LCAT activity with consumption of preβ_1-HDL. For CAD patients, the increased PLTP activity does not match the decreased LCAT activity. It may be speculated further that PLTP-generated preβ_1-HDL, not participating efficiently in their metabolism by low LCAT activity in CAD, is increasingly cleared from the circulation because of low *CUBN* gene expression. Of note, there is a nonconclusive opinion that PLTP activity is a risk factor for human CAD with an unclear effect on cholesterol efflux [44]. Positive association of *HMGCR* expression with the *D* parameter only for CAD patients originates from the inverse correlations of *HMGCR* expression with plasma HDL-C, pHDL-C:apoA-I, and pHDL-PL:A-I (data not shown), which in turn are inversely correlated with the *D* parameter. Thus, HMGCR-induced increase of cholesterol synthesis in CAD is associated with the increase of apoA-I dissociation from HDL.

Third, lipid-free/lipid-poor apoA-I that may originate from the HDL-bound state, both for CAD and control HDL, seems to be equally efficient in ABCA1-mediated efflux. The two dependencies in the double reciprocal plot intersected in the upper right corner (Fig. 3), which is definite evidence of competition of two substrates for a single enzyme [23] with the relation V_1 (preβ-HDL) $>$ V_2 (α-HDL), which is our case. The competition becomes more prominent with the increase of the α-apoA-I:preβ-apoA-I ratio *R* (13.0 in this case). The above-metioned low *CUBN* gene expression in CAD may underlie the increased clearance of PLTP-generated preβ_1-HDL. We describe for the first time the competition between preβ-HDL and α-HDL for ABCA1 when both substrates during kinetic measurements are present at the same fixed ratio *R* as in plasma. Two efflux kinetics have to be measured separately: the first with fully denatured HDL and the second with intact HDL. The ability of isolated apoA-I [3,45] and HDL fractions [3,13,46] to interact mainly with ABCA1 has been described with conflicting results. However, the absence of both the second substrate and appropriate kinetic treatment largely hindered competition visualization, while only correlations with the level and composition of other lipoproteins were being analyzed. The obtained V_m and K_m values were lower than the analogous mean values (22.3% and 0.64 µg/mL) for ABCA1-mediated efflux to lipid-free apoA-I for 6 h [3]. Of note, only 40–60% of apoA-I molecules were dissociated in water upon pHDL denaturation by 4.25 M urea (compared to 10–20% of preβ-apoA-I in intact HDL), and V_{m} would not reach the maximal limiting value for lipid-free apoA-I due to the lower V_{m} value for remaining lipid-bound apoA-I. However, similar efflux kinetics at two zones of denaturation transition largely exclude this possibility. Of note, the structure of residual HDL with lipid-bound apoA-I, still existing at the transition midpoint, is perturbed by urea, which excludes the possibility of accepting cholesterol by residual HDL.

4.3 (Patho)physiological Significance

The decreased ABCA1-mediated efflux efficiency of intact HDL as a mixture of lipid-free and lipid-bound apoA-I in CAD has been described [17,18]. Despite the attempts to normalize efflux to preβ_1 levels as an indicator of lipid-poor apolipoprotein, the conventional CEC measurements were performed with HDL as a mixture of lipid-free and lipid-bound apoA-I without the separation of individual efflux reactions with different acceptors [17,18,47]. However, the overall reaction velocity for a two-substrate reaction with a single enzyme depends on the concentrations of both substrates depicted by R values (Eqn. 8), and the numerical simulation of this dependency revealed a non-linear increase of the V_m value with the decrease of the R value. The effective value of the maximal reaction velocity V_m, 7.9%/2 h, for intact HDL from CAD patients with low HDL-C levels and with the mean R value of 13.0 lies between the V_1 (17.5 %/2 h) and V_2 (5.3%/2 h) limits. It can be speculated further that the combined assessment of lipid-free and HDL-bound apoAI could improve CAD risk prediction and clarify the existing inconsistencies in HDL functionality in cholesterol efflux, in particular, the significance of large [47] and small [3] HDL. Also, the combined assessment of two substrates could localize more exactly the impairment of cholesterol efflux by oxidative modification of HDL in CAD to lipid and/or protein moieties [48,49]. In any case, the obtained data strongly suggest the integrity of the structure of lipid-free apoA-I in both control and CAD patients. However, we cannot exclude the possibility of oxidative modification of lipids in HDL. The exact nature of the dependency of V_m on R for control and CAD patients with different HDL-C levels and the contribution of HDL fractions is a matter of ongoing experiments based on the application of the kinetics of lipid-free and HDL-bound apoA-I competing with each other for the ABCA1 transporter. We suggest that this dependency may underlie the diminished cholesterol efflux in CAD.

5. Limitations

Limitations include modest sample size, use of only RAW 264.7 murine macrophages, restriction of urea-induced denaturation as an *in vitro* model, and causal contribution of apoA-I dissociation to CAD development.

6. Conclusions

For CAD patients, apoA-I partitions more into the water phase at urea-induced HDL denaturation due to the higher free energy of apolipoprotein in the lipid phase with the negative correlations of the dissociation parameter with absolute and normalized per apoA-I cholesterol and phospholipid levels in HDL particles. For control patients, the dissociation of apoA-I in the water phase is associated with *ABCA1* and *TLR8* gene expression. The upregulated expression of *CETP*, *LPL*, and *PLTP* genes and the downregulated expression of the *LCAT* gene in CAD are suggested to result in the lipid enrichment of HDL. *PLTP* gene expression may underlie the increased apoA-I dissociation in CAD. The total initial rates of ABCA1-mediated cholesterol efflux from RAW 264.7 macrophages, when preβ-HDL and α-HDL act as competitive inhibitors of each other for the binding to the ABCA1 transporter, were measured independently with intact HDL and pre-denatured HDL as a source of lipid-free apoA-I. The retained CEC of lipid-free/lipid-poor apoA-I in CAD may be masked by the higher competition of α-HDL, which possesses lower CEC, with preβ-HDL for ABCA1 binding. This new approach may be used in the analysis of lipid-free and HDL-bound apoA-I as independent predictors of CAD.

Availability of Data and Materials

All raw data reported in this paper will also be shared by the lead contact upon request.

Author Contributions

ADD – concept of the study; writing text of the paper; supervision of the study; MAP – patient selection, coronary angiography, laboratory analysis, and search references; VBB – conducting experiments; preparation of figures and tables. ADD and VBB – analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The project was approved by ethics committee of the M.F. Vladimirsky Moscow Regional Research and Clinical Institute MONIKI (protocol no. 12479/2019, February 17, 2019). All involved patients provided voluntary informed consent to participate in the study.

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Conflict of Interest

The authors declare no conflict of interest.

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