Plasma cholesterol homeostasis, HDL remodeling and function during the acute phase reaction[s]

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Abstract   Acute phase reaction (APR) is a systemic inflammation triggered by several conditions associated with lipid profile alterations. We evaluated whether APR also associates with changes in cholesterol synthesis and absorption, HDL structure, composition, and cholesterol efflux capacity (CEC). We analyzed 59 subjects with APR related to infections, oncologic causes, or autoimmune diseases and 39 controls. We detected no difference in markers of cholesterol synthesis and absorption. Conversely, a significant reduction of LpA-I- and LpAI:AII-containing HDL (−28% and −44.8%, respectively) and of medium-sized HDL (−10.5%) occurred in APR. Total HDL CEC was impaired in APR subjects (−18%). Evaluating specific CEC pathways, we found significant reductions in CEC by aqueous diffusion and by the transporters scavenger receptor B-I and ABCG1 (−25.5, −41.1 and −30.4%, respectively). ABCA1-mediated CEC was not affected. Analyses adjusted for age and gender provided similar results. In addition, correcting for HDL-cholesterol (HDL-C) levels, the differences in aqueous diffusion total and ABCG1-CEC remained significant. APR subjects displayed higher levels of HDL serum amyloid A (+20-folds; *P* = 0.003).[JLR] In conclusion, APR does not associate with cholesterol synthesis and absorption changes but with alterations of HDL composition and a marked impairment of HDL CEC, partly independent of HDL-C serum level reduction.—Zimetti, F., S. De Vuono, M. Gomaraschi, M. P. Adorni, E. Favari, N. Ronda, M. A. Ricci, F. Veglia, L. Calabresi, and G. Lupattelli. Plasma cholesterol homeostasis, HDL remodeling and function during the acute phase reaction. *J. Lipid Res.* 2017. 58: 2051–2060.

Acute phase reaction (APR) can be defined as a complex response of the organism to an endogenous or exogenous stimulus inducing systemic inflammation. APR can be triggered by several pathological causes such as infections, traumas, myocardial infarction, connective tissue disorders and malignancies (1). During APR, several systemic and metabolic alterations occur, such as fever onset and elevation of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Also among the changes are increased serum levels of acute phase proteins such as fibrinogen, ferritin, serum amyloid A (SAA), α-2 globulins, and haptoglobin, as well as decreased serum albumin levels (1).

During APR, serum lipid profile undergoes typical modifications including reduced HDL-cholesterol (HDL-C) levels, often associated with total and LDL-cholesterol (LDL-C) decrease, and increase or no change in triglycerides (TGs) (2). The mechanisms underlying HDL-C serum level decrease during inflammation, likely to be multiple, are not entirely known. For example, it has been suggested that a reduction of liver ApoA-I secretion and an increase in endothelial lipase or phospholipase A2 expression may have a role (3). An alteration of plasma lipid profile may also derive from an inadequate balance between endogenous synthesis, absorption from gut, and clearance (4). To date,

no studies have evaluated whether an imbalance between cholesterol synthesis and absorption underlies HDL level reduction during APR.

During systemic inflammation, HDL particles undergo profound variations in terms of structure and composition. Some HDL components, such as ceruloplasmin and SAA, increase proportionally to the degree of inflammation (5, 6), whereas other molecules, such as paraoxonase 1, decrease (7). Finally, HDL functional properties are modified in APR, with a shift from an anti- to a pro-inflammatory phenotype (8). Among the protective functions of HDL is their cholesterol efflux capacity (CEC). Cell cholesterol efflux is the first limiting step of reverse cholesterol transport, the process promoting the transfer of excess cholesterol from peripheral tissues to the liver for final excretion (7, 9–11). HDL CEC has recently been proposed as a cardiovascular risk marker (12). Indeed, HDL CEC impairment has been described in inflammatory-based diseases such as rheumatoid arthritis, psoriasis, and systemic lupus erythematosus (13–15). Atherosclerosis itself is an inflammatory disease and its predisposing conditions, such as diabetes, obesity, and metabolic syndrome, are associated with a low-grade subclinical inflammation (16, 17).

Thus, the modifications in serum lipids associated to APR, particularly those regarding HDL structure, composition, and capacity to maintain cell cholesterol homeostasis, could explain at least in part the link between inflammation and atherosclerosis. Epidemiological studies have shown that patients suffering from chronic infections (1, 18, 19) or chronic inflammatory diseases, including rheumatoid arthritis (20), psoriasis (21), and systemic lupus erythematosus (22) have increased cardiovascular risk not fully explained by the traditional risk factors.

The aim of this work was to verify whether an unbalance between cholesterol synthesis and absorption associates with lipid profile changes in subjects with APR related to infections, oncologic causes, or autoimmune diseases. In addition, we evaluated whether APR is characterized by alterations of HDL structure, composition, and cell cholesterol efflux-promoting function. Finally, we looked for a possible relationship between HDL-related parameters, inflammatory status, and plasma markers of cholesterol homeostasis.

MATERIALS AND METHODS

Study subjects

We examined 59 subjects (39 males and 20 females, aged between 19 and 83 years old) who referred to the Internal Medicine Department of Perugia General Hospital and presented serum CRP levels >1.5 mg/dl. Laboratory parameters, including ESR, ferritin, white blood cells (WBCs), and external body temperature of the APR group, are shown in **Table 1**. APR was caused by infections (n = 37), oncologic causes (n = 8), and autoimmune diseases (n = 14). Among infections were erysipelas, infected wounds, pneumonia, urinary tract infections, abscesses, and pelvic infections. Oncologic causes included lymphoma and colon, pancreas, lung, or oral carcinomas. Patients with autoimmune diseases presented rheumatoid arthritis, systemic lupus erythematosus, Still disease, ulcerative colitis, Crohn's disease, polymyalgia rheumatica, ankylosing spondylitis, sarcoidosis, and overlap syndrome. Among APR subjects, the prevalence of cardiovascular disease-related conditions was 14.3% for type-2 diabetes, 32.1% for hypertension, and 25% for coronary heart disease. A control group of 39 subjects (11 males and 28 females, aged between 22 and 81 years old) who referred to the Internal Medicine Department of Perugia General Hospital without APR was included in the study. Laboratory parameters, including ESR, ferritin, WBCs, and external body temperature of the control subjects are shown in Table 1. This group included subjects with anxiety or depression, epilepsy, migraine, kidney gallstones without infection, osteoporosis, and hypertension. Among control subjects, the prevalence of cardiovascular disease-related conditions was 12.5% for type-2 diabetes, 43.2% for hypertension, and 10.8% for coronary heart disease.

To exclude the potential effect of the poor matching in terms of age and sex between APR patients and controls (Table 1), all of the statistical analyses were adjusted for these two parameters. Both P-values from unadjusted and adjusted analyses are reported in the Results section. Subjects taking medications interfering with lipid metabolism were excluded from the study. All subjects underwent clinical examination. BMI was defined as weight (kg)/squared height (m^2). Blood pressure was measured in triplicate by

TABLE 1. Clinical and laboratory parameters of control and APR subjects

	Control Subjects	APR Subjects	P	P Adjusted for Age and Gender
Age (y)	59 ± 15	63 ± 16	0.088	N/A
BMI	25.77 ± 4.97	24.59 ± 4.92	0.407	0.496
SBP (mmHg)	133.19 ± 17.95	125.49 ± 18.47	**0.033**	**0.009**
DBP (mmHg)	78.42 ± 10.72	75.09 ± 11.34	0.218	0.123
CRP (mg/dl)	0.1 (0.1–0.3)[a]	6 (2–15.7)[a]	**<0.001**	**<0.001**
ESR (mm/h)	22.18 ± 16.94	64.72 ± 33.75	**<0.001**	**<0.001**
WBC (×10^3/mmc)	7.55 ± 2.56	9.89 ± 5.26	**0.010**	**0.016**
Ferritin (ng/ml)	56.6 (24–96)[a]	185.4 (61.5–592.4)[a]	**<0.001**	**0.0007**
Temperature (°C)	36.30 ± 0.64	37.34 ± 1.07	**<0.001**	**<0.001**
Glicemia (mg/dl)	94.67 ± 21.50	95.26 ± 27.25	0.853	0.742
Insulinemia (mUI/l)	7.4 (5.4–10.6)[a]	4.4 (3.2–6.5)[a]	**0.003**	**0.049**
HOMA-IR (computer-based calculation)	1.01 (0.72–1.48)	0.64 (0.45–0.97)	0.480	0.100

Normally distributed data are expressed as mean ± SD; the nonparametric Mann-Whitney test was used to compare the two groups. P-values adjusted for age and gender were obtained by general linear models. P-values expressing significant differences are shown in bold. DBP, diastolic blood pressure; SBP, systolic blood pressure; HOMA-IR, Homeostasis Model Assessment-Insulin Resistance index; N/A, not applicable.
[a] Skewed data are expressed as median (interquartile range).

a physician with a mercury sphygmomanometer and a brachial cuff of appropriate size placed around the nondominant arm. Blood samples were drawn within 24 h from admission. CRP, ESR, WBC count, and ferritin were evaluated with an automated analyzer. Glycemia and serum insulin were evaluated by ELISA. The Homeostasis Model Assessment of Insulin Resistance was calculated with a computer-based model (23).

The study was performed in accordance with the ethical principles set in the Declaration of Helsinki for experiments involving humans. The study protocol received approval from the local ethical committee. Written informed consent was obtained from all study participants.

Lipid profile

Total cholesterol (TC), HDL-C, and TG were measured with an enzymatic colorimetric method. For HDL-C, the method was applied after precipitation of apoB-containing lipoproteins with polyethylene glycol (PEG). LDL-C was calculated by the Friedewald formula. apo AI and B were measured by immune nephelometry.

Cholesterol synthesis and absorption

The quantification of plasma noncholesterol sterols, i.e., lathosterol, sitosterol, and campesterol, allows the estimate of cholesterol synthesis and absorption. Lathosterol is considered a surrogate marker of cholesterol synthesis; campesterol and sitosterol are surrogate markers of cholesterol absorption (24).

Plasma lathosterol, campesterol, and sitosterol were measured by gas chromatography coupled to mass spectrometry with multiple selected ion monitoring, according to the protocol of Ahmida et al. (25). This procedure measures TC, lathosterol, campesterol, and sitosterol in the same run. The test on the procedure reproducibility, run with 10 samples in the same day for several days, showed coefficients of variation ranging from 1.5% to 2.4% within 1 day and from 2.8% and 3.4% among days. Noncholesterol sterols were standardized and expressed as ratios to cholesterol (mmol/mmol), to exclude the effect of different cholesterol levels, unless otherwise specified.

HDL composition and subclasses

The plasma concentration of HDL particles containing only apoA-I (LpA-I) and that of particles containing both apoA-I and apoA-II (LpA-I:A-II) was determined by electroimmunodiffusion in agarose gel (Sebia Italia). HDL distribution according to size was determined by nondenaturing polyacrylamide gradient gel electrophoresis of the d < 1.21 g/ml plasma total lipoprotein fraction (26). HDL subclasses were divided into three size intervals: small (diameter 7.2–8.2 nm), medium (diameter 8.2–8.8 nm), and large (diameter 8.8–12.7 nm) HDL. The protein-stained gels were scanned with an imaging densitometer and protein content evaluated separately for each HDL subclass. HDL subclasses were also analyzed by nondenaturing 2D-electrophoresis followed by immunodetection with a commercial polyclonal antibody against human apoA-I (Calbiochem) (27). Serum content of preβ-migrating HDL was expressed as percentage of total apoA-I signal.

HDL CEC

HDL CEC was measured in a subgroup of APR patients (n = 24) and control subjects (n = 25). Patient subset was representative of the main group in terms of APR causes (14 infectious, 4 oncologic, and six autoimmune). HDL CEC was evaluated through widely validated isotopic techniques by using specific cellular models to allow the analysis of the main pathways of cell cholesterol efflux to HDL. First, J774 murine macrophages (J774 A.1, from ATCC) in basal conditions were used as a model of

cholesterol aqueous diffusion (AD); J774 cells incubated with 0.3 mM of a cAMP analog (cpt-cAMP, Sigma-Aldrich, Milano, Italy) to induce ABCA1 expression, were used as a model of total CEC as previously described (28, 29). The net contribution of ABCA1-mediated cholesterol efflux was calculated as the difference between total CEC and AD-CEC (30). Rat hepatoma Fu5AH cells, in the absence or presence of a specific SR-BI inhibitor (Block Lipid Transfer-1 10 μM, ChemBridge, San Diego, CA), were used to evaluate SR-BI-mediated cholesterol efflux (31). CHO cells, transfected or not with the human ABCG1 gene, were used to evaluate ABCG1-mediated cholesterol efflux. The specific ABCG1 contribution was calculated as the difference between CEC in ABCG1-transfected and nontransfected cells (32). In all assays, the cells were initially labeled with [1,2-3H]cholesterol (PerkinElmer, Milano, Italy) for 24 h in the presence of an ACAT inhibitor to keep all cholesterol in the unesterified form. After an equilibration period in medium containing 0.2% BSA (BSA, Sigma-Aldrich), cells were exposed for 4 h to 2% HDL serum fractions for SR-BI, total, AD, and ABCA1-mediated CEC, and for 6 h to 1% HDL serum fractions for ABCG1-mediated CEC. Serum HDL fractions were obtained from whole serum by precipitation of the apoB-containing lipoproteins with PEG, as previously described (33). HDL CEC was expressed as a percentage of the radioactivity released into the medium over the total radioactivity incorporated by cells. A pool of human normo-lipidemic sera was tested in each assay as reference standard 1 and its CEC was used to normalize the patient samples values from different experiments to correct for the inter-assay variability. A second pool of human normo-lipidemic sera as reference standard 2 was tested in each assay and its CEC, after normalization, was the index of the intra-assay variability.

SAA content in HDL

To evaluate SAA content in the HDL fraction, sera were depleted of the apoB-containing lipoproteins by precipitation with PEG (34). SAA was measured by ELISA assay (Novex by Life Technologies, Frederick, MD) in the same subset of samples analyzed for HDL CEC (n = 24 APR and n = 25 controls).

Statistical analysis

Data are reported as mean ± SD. Variables with skewed distribution (TG, CRP, ferritin, insulinemia, Homeostasis Model Assessment-Insulin Resistance index, and SAA) are reported as median and interquartile range and log-transformed before analysis. Univariate comparisons between APR and controls were performed by two-sided nonparametric Mann-Whitney test. Multivariate comparisons adjusted for age and sex were performed by general linear models. CEC results were further adjusted for HDL-C levels. Comparison of values among the three subgroups of APR causes (infectious, oncologic, and autoimmune) was performed by Kruskal-Wallis test. Associations between variables were assessed by Spearman correlation. *P*-values below 0.05 were considered as significant. All analyses were performed using the SAS Statistical package v. 9.4 (SAS Institute Inc.).

RESULTS

Detailed characteristics of patients and controls are reported in Table 1. Patients displayed a moderate increase in typical APR-defining parameters, which were significantly higher as compared with controls: CRP (mean + 19.5-fold, $P < 0.001$), ESR (mean + 2.9-folds, $P < 0.001$), WBCs (mean + 1.3-fold, $P < 0.01$), ferritin (mean + 6-fold, $P < 0.001$), and temperature (mean + 1.03-fold, $P < 0.001$) (Table 1).

TABLE 2. Serum lipid profiles of control and APR subjects

	Control Subjects	APR Subjects	P	P Adjusted for Age and Gender
TC (mg/dl)	194.25 ± 43.65	136.53 ± 37.59	**<0.001**	**<0.001**
LDL-C (mg/dl)	120.60 ± 36.36	82.50 ± 29.60	**<0.001**	**<0.001**
HDL-C (mg/dl)	52.20 ± 19.17	29.40 ± 11.38	**<0.001**	**<0.001**
TG (mg/dl)	100.5 (67.5–131.5)[a]	103.5 (77–150)[a]	0.186	0.190
apoA-I (mg/dl)	129.94 ± 30.92	84.03 ± 29.85	**<0.001**	**<0.001**
apoB (mg/dl)	73.85 ± 15.13	68.06 ± 20.35	0.139	0.795

Normally distributed data are expressed as mean ± SD; the nonparametric Mann-Whitney test was used to compare the two groups. *P*-values adjusted for age and sex were obtained by general linear models. *P*-values expressing significant differences are shown in bold.

[a] Skewed data are expressed as median (interquartile range).

Statistical analyses without and with adjustment for age and gender provided similar results. CRP levels were significantly different across the three groups of diagnoses (supplemental Table S1). In particular, infections were associated with higher CRP values compared with other diagnoses (mean + 1.95-fold and + 1.96-fold vs. autoimmune and oncologic causes, respectively). Conversely, we did not observe differences across diagnosis in the other inflammatory parameters examined.

Lipid profile

APR subjects showed an altered serum lipid profile characterized by reduced TC (−29.7%), LDL-C (−31.6%), and HDL-C (−43.7%) (**Table 2**). TG did not differ between the two groups. ApoA-I levels were markedly lower in APR subjects (−35.1%), whereas apoB levels were only slightly and nonsignificantly decreased compared with controls (Table 2). Analyses without and with adjustment for age and gender provided the same results. Stratifying patients according to the etiopathogenesis of APR, we did not detect significant differences in lipid changes except for lower apoA-I levels in infectious compared with oncologic conditions (mean – 28.5%, supplemental Table S2).

Cholesterol synthesis and absorption

As shown in **Table 3**, no difference was detected between APR and control groups in plasma markers of cholesterol synthesis and absorption, expressed as ratios over cholesterol. Similarly, we did not find differences in the ratio campesterol/lathosterol parameter that simultaneously reflects absorption and synthesis. Analyses without and with adjustment for age and gender provided the same results. In addition, no differences were found across the three groups of causes of APR (supplemental Table S3).

HDL composition and size

Looking in detail at the HDL particles composition and size, we found that both LpA-I and LpAI:AII were significantly reduced in APR subjects compared with controls, with a greater reduction for LpAI:AII (−28% and −44.8% for LpA-I and LpA-I: A-II, respectively, **Table 4**). Percentage of small and large HDL did not differ between the two groups. Conversely, medium-sized HDL particles were slightly but significantly reduced in inflamed subjects (−10.5%). Preβ HDL content was lower in APR subjects (−26%; Table 4). The same results were obtained analyzing data without and with adjustment for age and gender except for the difference in preβ HDL levels, which was lost after adjustment. Stratifying APR patients based on the diagnosis, we found a trend toward significant difference across groups in terms of LpA-I:A-II and a significant difference in preβ HDL (supplemental Table S4). In particular, APR due to infections associated with the lowest levels of both LpA-I:A-II (mean − 37% and − 17.3% vs. autoimmune and oncologic causes, respectively) and preβ HDL (mean − 57.7% and − 66.7% vs. autoimmune and oncologic causes, respectively).

HDL CEC

We next evaluated HDL CEC based on previous observations of compromised HDL functional properties in inflammatory conditions (13, 14, 34, 35). We measured serum HDL CEC by distinguishing between the main cell cholesterol efflux pathways in 24 subjects with APR and 25 controls. As shown in **Fig. 1A**, cholesterol efflux from cAMP-stimulated J774 macrophages (total CEC) was significantly lower in APR subjects compared with controls (−18%). Such difference persisted after adjustment for age, gender, and HDL-C levels (Model 3, **Table 5**). Separately analyzing

TABLE 3. Non-cholesterol plasma sterols in control and APR subjects

	Control Subjects	APR Subjects	P	P Adjusted for Age and Gender
Lathosterol (10^2 μmol/mmol cholesterol)	109.19 ± 57.71	106.95 ± 67.51	0.727	0.883
Campesterol (10^2 μmol/mmol cholesterol)	36.05 ± 19.57	33.85 ± 20.07	1.000	0.465
Sitosterol (10^2 μmol/mmol cholesterol)	92.59 ± 56.53	82.13 ± 42.49	0.793	0.351
Campesterol/lathosterol ratio	0.51 ± 0.61	0.52 ± 0.59	0.872	0.656
Sitosterol/lathosterol ratio	1.18 ± 1.11	1.15 ± 1.06	0.817	0.546

Data are expressed as mean ± SD; the nonparametric Mann-Whitney test was used to compare the two groups. *P*-values adjusted for age and sex were obtained by general linear models.

TABLE 4. HDL composition and size in control and APR subjects

	Control Subjects	APR Subjects	P	P Adjusted for Age and Gender
LpA-I (mg/dl)	47.00 ± 14.83	33.85 ± 11.53	**<0.001**	**0.015**
LpA-I:A-II (mg/dl)	89.04 ± 22.68	49.08 ± 25.47	**<0.001**	**<0.001**
Small HDL (%)	20.32 ± 6.90	19.49 ± 8.54	0.563	0.435
Medium HDL (%)	17.84 ± 3.87	15.97 ± 2.84	**0.048**	**0.019**
Large HDL (%)	61.84 ± 9.44	63.20 ± 13.35	0.204	0.172
Pre-β HDL (%)	9.36 ± 5.24	6.94 ± 5.50	**0.034**	0.374

Data are expressed as mean ± SD; the nonparametric Mann-Whitney test was used to compare the two groups. *P*-values adjusted for age and sex were obtained by general linear models. *P*-values expressing significant differences are shown in bold.

the two cholesterol efflux pathways contributing to total CEC, we found that AD was remarkably reduced in APR patients as compared with controls (−25.5%; Fig. 1B), whereas ABCA1-mediated CEC did not significantly differ between groups (Fig. 1C). As for total CEC, the difference in AD CEC between APR and control groups remained significant after adjusting for age, gender, and HDL-C levels (Model 3, Table 5). HDL CEC via the SR-BI and ABCG1 transporters was also impaired in subjects with APR, with SR-BI being reduced to greater extent (−41.1% and −30.4% for SR-BI and ABCG1, respectively) (**Fig. 2A**, B). The differences in SR-BI- and ABCG1-mediated CEC between groups were significant both without and with adjustment for age and gender (Model 2, Table 5). After further adjustment for HDL-C levels, the difference between groups was lost for SR-BI-mediated CEC and conserved for the ABCG1 pathway (Model 3, Table 5). Due to the small number of samples analyzed for CEC, sub-analysis for APR causes was not possible.

SAA content in HDL

The amount of SSA associated with HDL was significantly increased in APR subjects compared with controls; median and interquartile range was 25.59 (9.8–117.1) and 532 (69.19–2,322) in controls and APR, respectively (**Fig. 3**, P = 0.003). This difference remained significant after adjustment for age and gender (P = 0.004).

Relationship between inflammation, cholesterol synthesis, absorption, and HDL-related parameters

Finally, we looked at the possible relationship between the evaluated parameters. Analyzing controls and APR patients together, we found that the ABCG1-mediated CEC inversely correlated with the markers of inflammatory status (supplemental Fig. S1). In addition, the inflammatory values ESR and CRP inversely correlated with medium-sized HDL particles (r = −0.336; P = 0.008). Finally, SAA levels inversely correlated with ABCG1-mediated CEC (supplemental Fig. S2). All these correlations were lost when the two groups were analyzed separately.

We did not find significant correlations between the markers of cholesterol synthesis or absorption and the inflammatory status, either considering all subjects together or separately analyzing the two groups (data not shown). We did not find significant correlations of inflammatory indexes with HDL particle concentration, structure, and function in control subjects. However, some correlations were found within APR group (**Table 6**): CRP and ferritin levels

inversely correlated with HDL-C, apoA-I, and with LpAI:A-II levels. In addition, CRP directly associated with large HDL levels and with HDL SAA content. Again, only in the APR group, we observed an inverse and significant correlation between SR-BI-mediated CEC and body temperature (r = −0.566; P = 0.006, Table 6). Both SR-BI-mediated CEC and body temperature weakly correlated with plasma
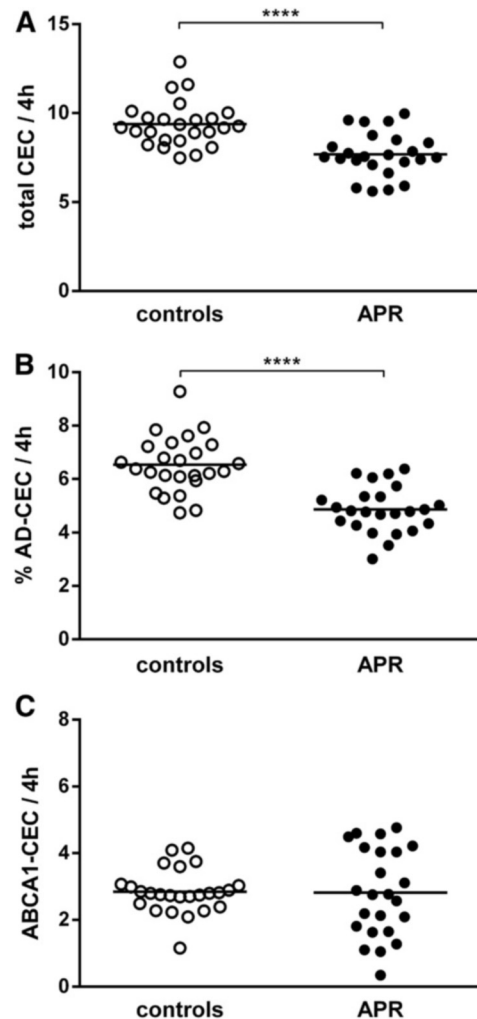

Fig. 1. HDL CEC: total (A), AD (B), and ABCA1-mediated CEC (C). Values are expressed as a percentage of radioactive cholesterol in cell supernatants after 4 h of exposure to apoB-depleted sera from controls (n = 25) and APR subjects (n = 24) over total radioactive cell cholesterol. Each point represents the mean CEC relative to a sample run in triplicate. Statistical significance was calculated by using a two-sided nonparametric Mann-Whitney test. ****P< 0.0001.

TABLE 5. HDL CEC in control and APR subjects

	Control Subjects	APR Subjects	P		
			Model 1	Model 2	Model 3
Total CEC (%)	9.38 ± 1.26	7.68 ± 1.24	**0.0001**	**0.0001**	**0.011**
AD-CEC (%)	6.53 ± 1.03	4.86 ± 0.86	**<0.0001**	**<0.0001**	**0.013**
ABCA1-CEC (%)	2.84 ± 0.66	2.82 ± 1.32	0.881	0.772	0.370
SR-BI-CEC (%)	1.97 ± 0.64	1.16 ± 0.52	**0.0001**	**0.0001**	0.930
ABCG1-CEC (%)	5.85 ± 0.93	4.07 ± 1.16	**<0.0001**	**<0.0001**	**<0.0001**

Data are expressed as mean ± SD; Model 1: unadjusted; Model 2: adjusted for age and gender; Model 3: adjusted for age, gender, and HDL-C. Adjusted P-values were obtained by general linear models. P-values expressing significant differences are shown in bold.

HDL-C levels ($r = 0.403$, $P = 0.051$ and $r = -0.239$, $P = 0.091$ for SR-BI CEC and body temperature, respectively) and more markedly with apoA-I levels ($r = 0.7102$; $P = 0.0001$ and $r = -0.299$; $P = 0.033$ for SR-BI CEC and temperature, respectively).

We did not find any significant associations between the plasma markers of cholesterol synthesis and absorption and the HDL-related parameters except for an inverse correlation observed in control subjects between campesterol and small and medium HDL ($r = -0.4312$; $P = 0.045$ and $r = -0.4435$; $P = 0.0436$ with small and medium HDL, respectively), and a positive relationship with large HDL ($r = 0.434$; $P = 0.044$). These relationships were absent in APR subjects.

DISCUSSION

The present results show, for the first time, that APR, characterized by a strong alteration of quantitative lipid
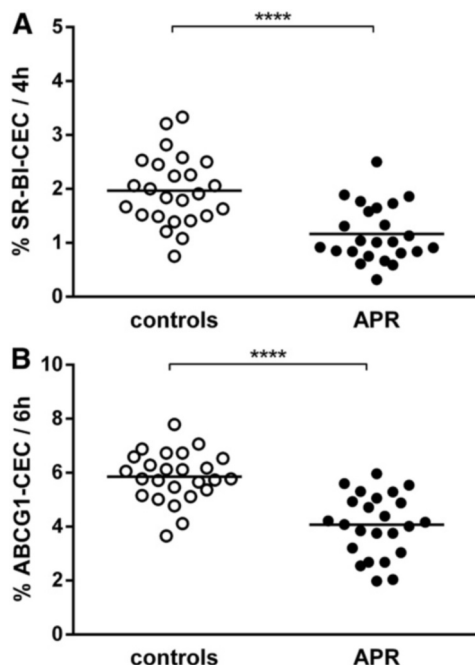

Fig. 2. HDL CEC: SR-BI- (A) and ABCG1-mediated CEC (B). Values are expressed as a percentage of radioactive cholesterol in cell supernatants after 4/6 h of exposure to apoB-depleted sera from both controls (n = 25) and APR subjects (n = 24) over total radioactive cell cholesterol. Each point represents the mean CEC relative to a sample run in triplicate. Statistical significance was calculated by using a two-sided nonparametric Mann-Whitney test. ****$P < 0.0001$.

profile, is not accompanied by significant variations in either cholesterol synthesis or absorption. Conversely, this condition is associated to HDL composition and size modifications and to profound impairment of the HDL atheroprotective CEC.

The first aim of our study was to investigate whether an impaired balance between cholesterol synthesis and absorption could play a role in lipid serum level changes during APR. In fact, the balance between hepatic cholesterol synthesis and intestinal cholesterol absorption, studied by quantifying the noncholesterol sterols, is lost in several pathologic conditions leading to dyslipidemia and increased cardiovascular risk (4, 36). In general, inflammatory stress has been associated with reduced total, LDL, and HDL cholesterolemia (2) and our data are in line with these modifications. However, available data on the mechanisms by which inflammation affects cholesterol homeostasis are conflicting; for example, the inflammatory cytokines TNF-α, TNF-β, IL-1, and IFN-γ have been shown to stimulate hepatic cholesterol synthesis in mice (37–39), whereas IL-1 inhibits cholesterol synthesis in human hepatoma HepG2 cells (40). Our in vivo human study indicates that impaired cholesterol synthesis is not involved in lipid profile alterations. Similarly, we found no differences in cholesterol absorption. This finding is somewhat surprising, as APR would be expect to be associated with a reduction in gut cholesterol absorption, due to the limited food intake secondary to the inflammatory state, at least before medical intervention. Thus, possible suggested mechanisms for APR-induced dyslipidemia could be an increased catabolism of cholesterol-rich lipoproteins or their retention outside the plasma, rather than an effect on cholesterol synthesis or absorption (2).

Due to the beneficial properties of HDL with respect to cardiovascular protection, we focused on HDL level, composition, and function. We found an inverse relationship between APR HDL-C level and the inflammatory parameters CRP and ferritin, consistent with previous findings (5, 8). The mechanisms leading to HDL-C level changes during APR are currently not fully understood, but several have been proposed: an increased hepatic scavenger receptor expression, leading to an enhanced turnover of HDL (41); a suppressed expression of the hepatic ABCA1 transporter, resulting in reduced HDL generation (42); a higher lipolytic activity of secretory phospholipase A$_2$ (43); and an increase in HDL-associated SAA, enhancing HDL clearance (44). The latter mechanism seems to be supported by the elevation of HDL-associated SAA in our APR subjects
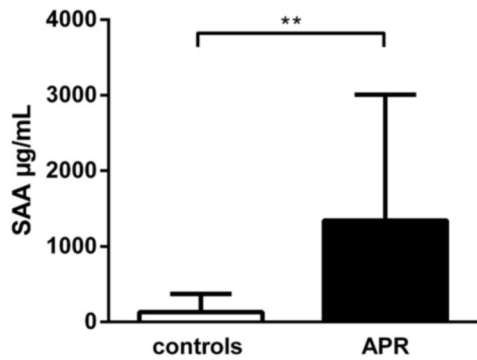
Fig. 3. HDL SAA content in controls (n = 25) and APR subjects (n = 24). Data are presented as mean ± SD. Statistical significance was calculated by using a two-sided nonparametric Mann-Whitney test. **$P = 0.003$

that was found directly correlated to CRP plasma concentration, as previously reported (45, 46).

With respect to the structural changes, the present results are consistent with our previous findings obtained in patients with APR due to acute myocardial infarction and consisting of the reduction of medium-sized HDL containing both apoA-I and apoA-II (45, 47). These data indicate that HDL distribution into distinct subclasses for size and composition is similarly affected by APR, independently of the triggering event.

The inverse correlation that we observed between the inflammatory status (CRP level) and medium-sized HDL containing both apoA-I and apoA-II further supports the

idea of a specific impact of inflammation on HDL subclass distribution. Notably, the decrease of HDL containing both apoA-I and apoA-II was slightly more evident in the group of patients with APR related to infections in which the inflammatory status was relatively more pronounced.

We observed a profound impairment of HDL CEC in the APR group. CEC is a measure of HDL anti-atherogenic properties, recently identified as a cardiovascular risk biomarker and as a promising target for prevention and therapy, independently of plasma HDL-C concentrations (48). Other studies already demonstrated an impairment of HDL CEC in several inflammatory diseases (13, 14, 35, 49). However, the novelty of our observation is that CEC is altered in a heterogeneous group of patients, having only inflammatory status in common.

In detail, we observed a specific impairment of the total CEC, the parameter associated with prevalent atherosclerosis and incident cardiovascular events (50, 51). Unfortunately, cardiovascular risk-related parameters were not available in our patients, but it is well known that conditions associated to inflammation are characterized by high cardiovascular risk (52). Indeed, elevated levels of the acute phase proteins, even for a limited span of time, are related to higher cardiovascular disease risk (53, 54). ABCA1-mediated CEC was not affected in our patients, consistent with previous results (6). The opposite finding of de La Llera Moya et al. (34) for this parameter may be due to the specificity of their research protocol, based on lipopolysaccharide (LPS) injection in healthy volunteers.

TABLE 6. Relationship between inflammatory parameters and HDL concentration, size, composition, and function in APR subjects

	ESR	CRP	WBC	Ferritin	Temperature
HDL-C	$r = -0.103$	**$r = -0.346$**	$r = -0.016$	**$r = -0.416$**	$r = -0.239$
	$P = 0.440$	**$P = 0.008$**	$P = 0.906$	**$P = 0.002$**	$P = 0.091$
apoA-I	$r = -0.071$	**$r = -0.485$**	$r = -0.019$	**$r = -0.344$**	**$r = -0.299$**
	$P = 0.596$	**$P = 0.001$**	$P = 0.887$	**$P = 0.010$**	**$P = 0.033$**
LpA-I	$r = 0.052$	$r = 0.052$	$r = -0.005$	$r = -0.158$	$r = -0.035$
	$P = 0.723$	$P = 0.728$	$P = 0.973$	$P = 0.300$	$P = 0.826$
LpA-I:A-II	$r = -0.067$	**$r = -0.587$**	$r = -0.170$	**$r = -0.310$**	**$r = -0.385$**
	$P = 0.653$	**$P < 0.001$**	$P = 0.248$	**$P = 0.038$**	**$P = 0.013$**
Small HDL	$r = -0.035$	$r = -0.240$	$r = -0.130$	$r = 0.004$	$r = -0.066$
	$P = 0.826$	$P = 0.136$	$P = 0.418$	$P = 0.979$	$P = 0.704$
Medium HDL	$r = -0.089$	$r = -0.293$	$r = -0.148$	$r = 0.009$	$r = -0.123$
	$P = 0.578$	$P = 0.067$	$P = 0.354$	$P = 0.959$	$P = 0.480$
Large HDL	$r = 0.098$	**$r = 0.336$**	$r = -0.211$	$r = 0.106$	$r = 0.164$
	$P = 0.531$	**$P = 0.029$**	$P = 0.175$	$P = 0.514$	$P = 0.333$
preβ HDL	$r = 0.0739$	$r = -0.185$	$r = -0.231$	$r = -0.088$	$r = -0.303$
	$P = 0.634$	$P = 0.234$	$P = 0.131$	$P = 0.582$	$P = 0.065$
Total CEC	$r = -0.048$	$r = 0.287$	$r = 0.053$	$r = 0.103$	$r = -0.182$
	$P = 0.824$	$P = 0.183$	$P = 0.806$	$P = 0.647$	$P = 0.418$
AD-CEC	$r = -0.188$	$r = -0.026$	$r = -0.019$	$r = -0.293$	$r = -0.231$
	$P = 0.378$	$P = 0.907$	$P = 0.929$	$P = 0.186$	$P = 0.301$
ABCA1-CEC	$r = 0.185$	$r = 0.355$	$r = 0.064$	$r = 0.225$	$r = 0.144$
	$P = 0.386$	$P = 0.096$	$P = 0.765$	$P = 0.314$	$P = 0.523$
SR-BI-CEC	$r = 0.039$	$r = -0.262$	$r = 0.003$	$r = -0.299$	**$r = -0.566$**
	$P = 0.854$	$P = 0.226$	$P = 0.987$	$P = 0.177$	**$P = 0.006$**
ABCG1-CEC	$r = -0.220$	$r = -0.061$	$r = -0.003$	$r = -0.094$	$r = -0.138$
	$P = 0.301$	$P = 0.781$	$P = 0.989$	$P = 0.676$	$P = 0.539$
SAA	$r = 0.046$	**$r = 0.521$**	$r = 0.299$	$r = 0.176$	$r = 0.421$
	$P = 0.830$	**$P = 0.011$**	$P = 0.156$	$P = 0.434$	$P = 0.051$

Relationship between parameters was calculated by nonparametric correlation (Spearman's coefficient reported). Statistically significant relationships are indicated in bold.

In our study, APR was characterized by a decrease in SR-BI- and ABCG1-mediated CEC. The SR-BI-mediated CEC impairment in APR is in accordance with the observation of de La Llera Moya et al. (34) on the LPS model. However, in our work, the differences in SR-BI CEC were lost after adjustment for HDL-C levels, confirming the strong dependence of this parameter on serum HDL concentrations (13, 55). The reduction of ABCG1-mediated CEC that we observed in APR is consistent with previous observations in postoperative patients (6, 56) and in patients with auto-immune diseases (13, 57). The statistical significance of ABCG1-mediated CEC reduction persisted after correction for HDL serum levels, consistent with the reported weak or absent relationship of this parameter to serum HDL-C levels (13, 58). The impact of inflammation on this efflux pathway seems to be confirmed by the observation that methotrexate, an anti-inflammatory agent widely used in rheumatoid arthritis patients, improves ABCG1-mediated CEC (59). Further supporting this concept, we found a significant correlation between this CEC pathway and CRP, ESR, and ferritin when analyzing all subjects of the study. Interestingly, in the paper by de La Llera Moya et al. (34), the LPS-induced inflammation did not associate with ABCG1-mediated CEC reduction. With the limitation of the very small number of samples analyzed for ABCG1-CEC in that work, both their data and ours suggest that the mechanisms inducing HDL dysfunction in patients with pathologic conditions in real life might be very complex. It may be hypothesized that the reduced total and ABCG1-mediated CEC found in our patients is due to peculiar variations of HDL composition in terms of protein or phospholipid cargo able to compromise the anti-atherogenic properties of HDL, including CEC through ABCG1 (11, 60). For example, the robust and inverse relationship that we found between HDL SAA and ABCG1-mediated CEC could reflect a cause-effect link between inflammation and this cell cholesterol efflux pathway. Further studies are needed to mechanistically explore this relationship.

The positive correlation detected between CRP and mature HDL particles seems to suggest a direct link between inflammation and HDL maturation, in contrast with previous observations (5). However, this correlation could be explained as a phenomenon secondary to the deprivation of small/medium HDL induced by inflammation. Indeed, total HDL levels are reduced proportionally to inflammation indexes. In any case, mature HDL particles showed an intrinsic functional impairment as demonstrated by the reduced capacity to accept cholesterol from the ABCG1 transporter, even after adjusting for serum HDL-C concentration.

This study has some limitations. First, the sample size is small and the matching between cases and controls in terms of age and especially of gender is poor. The lack of differences between data without and with correction for age and gender suggests that differences in these variables do not significantly affect our results although we cannot totally exclude the presence of other residual confounding factors.

Notably, only preβ HDLs appear to be dependent on age and gender, consistent with previous reports (61). Another possible limitation of the study is that some control subjects present cardiovascular risk factors, possibly influencing lipid metabolism. However, the main inclusion criterion for the recruitment of controls was the absence of inflammation in a "real life" situation. Further studies comparing APR to healthy subjects are needed to confirm our data. Among the limitations of this study is also the use of an indirect method for cholesterol synthesis and absorption measurement. To increase reliability of the results, at least on cholesterol absorption, we followed the recommendation to use two markers, sitosterol and campesterol, instead of one (62). Our findings require further confirmation through the stable isotope technique (63).

In conclusion, we demonstrated that APR does not associate with modifications in markers of cholesterol absorption and synthesis but with alterations of HDL metabolism and quality. In particular, we found changes in HDL structure and a marked impairment of the athero-protective cholesterol efflux-promoting function, independent of HDL-C reduction. Further studies are needed to elucidate the specific mechanisms underlying our findings.

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