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Effect of a short-term diet and exercise intervention on inflammatory/anti-inflammatory properties of HDL in overweight/obese men with cardiovascular risk factors

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Roberts, Christian K., Carey Ng, Susan Hama, Anna Jane Eliseo, and R. James Barnard. Effect of a short-term diet and exercise intervention on inflammatory/anti-inflammatory properties of HDL in overweight/obese men with cardiovascular risk factors. *J Appl Physiol* 101: 1727–1732, 2006. First published August 10, 2006; doi:10.1152/jappphysiol.00345.2006.—There is significant debate regarding high-density lipoprotein cholesterol (HDL-C) and high-fiber, low-fat diets. The present study was designed to examine the effects of lifestyle modification on the inflammatory/anti-inflammatory properties of HDL in obese men ($n = 22$) with metabolic syndrome factors. Subjects were placed on a high-fiber, low-fat diet in a 3-wk residential program where food was provided ad libitum and daily aerobic exercise was performed. Fasting blood was drawn pre- and postintervention for serum lipids, lipid hydroperoxides, and the ability of subject HDL to alter low-density lipoprotein (LDL)-induced monocyte chemotactic activity (MCA) in a human artery wall coculture. Induction of MCA by control LDL in the absence of HDL was normalized to 1.0. Values >1.0 after HDL addition indicated proinflammatory HDL; values <1.0 indicated anti-inflammatory HDL. In addition, proteins involved in regulating HDL function, apolipoprotein A-I (apoA-I), paraoxonase 1 and 3, and platelet-activating factor acetylhydrolase were measured. After 3 wk, decreases in total cholesterol, LDL-cholesterol, HDL-C, triglycerides, total cholesterol-to-HDL cholesterol ratio, and lipid hydroperoxides (all $P < 0.05$) were noted. The HDL inflammatory index decreased ($P < 0.05$) from pro- (1.14 ± 0.11) to anti-inflammatory (0.94 ± 0.09). ApoA-I level and paraoxonase activity did not change; however, platelet-activating factor acetylhydrolase activity increased ($P < 0.05$). Despite a quantitative reduction in HDL-C, HDL converted from pro- to anti-inflammatory. These data indicate that intensive lifestyle modification improves the function of HDL even in the face of reduced levels, suggesting increased turnover of proinflammatory HDL.

atherosclerosis; lipids; apolipoprotein A-I; paraoxonase; platelet-activating factor acetylhydrolase; high-density lipoprotein

EARLY EPIDEMIOLOGICAL AND clinical studies established a link between dietary saturated fat, dietary cholesterol, serum cholesterol [especially low-density lipoprotein-cholesterol (LDL-C)] and coronary artery disease (CAD) mortality (7, 16, 37). Conversely, high levels of high-density lipoprotein cholesterol (HDL-C) appear to be protective, and the National Cholesterol Education Program (NCEP) has emphasized increasing HDL-C (9). Interest in high-fiber, low-fat diets has been tempered by data demonstrating that they lower the plasma HDL-C level (19). Framingham data noted $>40\%$ of events occurred in men and women with HDL-C levels ≥ 40 and ≥ 50 mg/dl, respectively (15). Thus a large portion of events occur

in patients with normal LDL-C and HDL-C levels (7), and there is a need for markers with enhanced individual predictive value (32).

HDL possesses many antiatherogenic properties, including roles in reverse cholesterol transport, decreasing LDL oxidation, and decreasing adhesion molecule expression (20). In this regard, it has been postulated that the ability of HDL to protect LDL against oxidation, termed the HDL inflammatory/anti-inflammatory properties, may be as important as its antiatherogenic role in reverse cholesterol efflux (21, 22, 43), and be more important than the level of HDL-C (1).

Our laboratory previously noted quantitative reductions in HDL-C following high-fiber, low-fat diet and exercise interventions (33, 34). It is possible that HDL inflammatory properties may be altered by lifestyle independent of the quantitative reduction in HDL-C, and thus the present study was designed to investigate this possibility in overweight/obese men with metabolic syndrome factors. Specifically, we had two hypotheses: 1) despite a quantitative reduction in HDL, subject HDL would induce less monocyte chemotactic activity (MCA) in vitro, indicating less pro-inflammatory HDL-C, and 2) the improvement in HDL inflammatory index would be associated with changes in protein levels and/or enzyme activities found in the HDL particle.

METHODS

Diet and exercise intervention. The study protocol was approved by the Human Subjects Protection Committee of the University of California, Los Angeles, and informed consent of all participating subjects was obtained. Serum samples for this study were obtained from 22 men (age range 46–76 yr, mean 62.8 yr) who voluntarily participated in the Pritikin Longevity Center 21-day residential diet and exercise intervention during 2001. All participants were overweight or obese [mean body mass index (BMI) = 33 ± 1 kg/m²] and 15 of the 22 had the metabolic syndrome according to the World Health Organization-modified criteria (18). Three subjects had diagnosed CAD, and the four others had one or two metabolic syndrome factors. Additionally, 1 subject was on statin therapy. All subjects were free of any viral infections and were able to consume the prescribed diet and to perform physical activity during the study. These subjects also took part in a previously reported study (34).

Once enrolled in the program, participants underwent a complete medical history and physical examination before the 21-day diet and exercise intervention as previously described (33). From dietary analysis software, prepared meals contained 12–15% of calories from fat (polyunsaturated-to-saturated fatty acid ratio = 2.4:1), 15–20% of calories from protein, and 65–70% of calories from primarily unre-

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finer carbohydrate, high in dietary fiber (>40 g/day). The program was designed to allow the subjects ad libitum eating without control of calories, only restricting animal protein. Carbohydrates were primarily in the form of high-fiber whole grains (≥ 5 servings/day), vegetables (≥ 4 servings/day), and fruits (≥ 3 servings/day). Protein was from plant sources, nonfat dairy (up to 2 servings/day), and fish/fowl (3.5-oz portions 1 day/wk and in soups or casseroles 2 days/wk). Saturated and trans-fatty acid intake was minimal, and added fats and sugars were not included.

Before starting the exercise training, subjects underwent a graded treadmill stress test according to a modified Bruce protocol to determine the appropriate individual level of exercise intensity. Based on the results, the subjects were provided with an appropriate training heart rate value and given an individualized walking program. The exercise regimen consisted of daily treadmill walking at the training heart rate for 45–60 min. The training heart rate was defined as 70–85% of the maximal heart rate attained during the treadmill test.

Twelve-hour fasting blood samples were drawn from the subjects in vacutainers (Becton-Dickinson Vacutainer Systems) containing SST clot activating gel between 6:30 and 8:00 AM on *days 1* and *21* of the intervention. The blood was transported on ice to the laboratory, and the serum was separated by centrifugation and stored at -80°C until analyzed. Weight was measured using a scale from Pennsylvania Medical Scales (model 7500). Height was measured using a stadiometer from Seca, attached to the wall. BMI was calculated as weight (kg)/height (m^2).

Determination of serum lipids. Total cholesterol, triglycerides (TG), and HDL-C were measured at a national commercial laboratory (Quest Diagnostics, Los Angeles, CA) using standardized techniques. The LDL-C was calculated as described by Friedewald et al. (13). Apolipoprotein A-I (apoA-I) was determined by an ELISA kit from Alerchek (Portland, ME); this assay has a coefficient of variation of 6–10%.

Measurement of lipid hydroperoxides. For quantitation of lipid hydroperoxides, serum lipids were extracted with chloroform-methanol, and hydroperoxides were quantitated by the method described by Auerbach et al. (3). Previously, van Lenten et al. (41) have noted this assay to be significantly correlated ($r^2 = 0.99$) with F2-isoprostane concentrations (41) and the coefficient of variation for this assay is well below 10%.

Inflammatory/anti-inflammatory properties of HDL. Human aortic endothelial cells (HAEC) were cultured as previously described (25, 40). In brief, the cells were subcultured and grown to confluence in 75-cm² flasks in M199 medium (Invitrogen) supplemented with 20% FBS, 0.8 ml 100 ml heparin, 2 mg endothelial cell growth factor-100 ml media (Becton-Dickinson), 1% penicillin-streptomycin-glutamine (Gibco BRL), and 1% sodium pyruvate (Gibco BRL). Human aortic smooth muscle cells (HASMC) were cultured in the same media without addition of endothelial cell growth factor or heparin. Wells in a 96-well plate were treated with 0.1% gelatin for at least 1 h. HASMC were seeded in the wells at a density of $\sim 5 \times 10^4$ cells/cm² and were cultured for 2–3 days at 37°C, 5% CO₂ at which time they had reached confluency. HAEC were subsequently overlaid on top of HASMC at $\sim 1 \times 10^5$ cells/cm² and were allowed to grow, forming a complete monolayer of confluent HAEC in 2 day. Lipoproteins (isolated by fast-performance liquid chromatography) and monocytes from healthy normal individuals were isolated by a modification of the Recalde procedure (10), and MCA was determined using standard control LDL in the absence or presence of the subjects' HDL (250 $\mu\text{g}/\text{ml}$, by protein) as previously described (22, 25). Briefly, pre- and postintervention HDL was added to confluent monolayers of HAEC. Eighteen hours later, cocultures were washed, and fresh culture medium without additions was added and incubated for an additional 8 h. This allowed the MCA released by the cells after stimulation by the oxidized LDL to be collected. Supernatants were collected and tested for MCA as a result of stimulation by the oxidized LDL (22). The supernatants were added to a standard Neuroprobe chamber

Table 1. Anthropometric and metabolic parameters of subjects undergoing a 21-day diet and exercise intervention

	Preintervention	Postintervention	% Decrease
No. with metabolic syndrome	15	6	60.0
No. obese (BMI >30 kg/m ²)	16	14	12.5
No. diabetic	4	2	50.0
Body weight kg	103.4 (22.9)	100.1 (23.0)†	3.2
BMI	33.1 (7.1)	32.1 (7.2)†	2.8
Total cholesterol mg/dl	206.8 (40.8)	163.9 (38.1)†	20.8
LDL-C, mg/dl	126.1 (38.5)	93.8 (33.8)†	25.6
HDL-C, mg/dl	43.7 (10.4)	39.4 (10.1)*	10.0
ApoA-1, mg/dl	21.9 (6.0)	20.9 (4.5)	4.4
Total cholesterol HDL-C	4.93 (1.26)	4.32 (1.10)*	12.3
LDL-C/HDL-C	2.92 (0.83)	2.44 (0.75)†	16.5
TG, mg/dl	219 (137)	155 (89)*	28.8

Values are means \pm SD for 22 Subjects. BMI, body mass index; LDL-C = low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoA-1, apolipoprotein, A-I; TG, triglycerides. * $P < 0.05$; † $P < 0.01$ postintervention vs. preintervention.

(NeuroProbe, Cabin John, MD), with isolated human peripheral blood monocytes added to the top. The chamber was incubated for 60 min at 37°C. After the incubation, the chamber was disassembled and the nonmigrated monocytes were wiped off. The membrane was then air dried and fixed with 1% glutaraldehyde and stained with 0.1% crystal violet dye. The number of migrated monocytes was determined microscopically and expressed as the means (SD) of 12 standardized high power fields counted in quadruple wells. Values in the absence of HDL were normalized to 1.0 and termed the HDL inflammatory index. Values >1.0 after the addition of HDL indicated pro-inflammatory HDL; values <1.0 indicated anti-inflammatory HDL. For this study, the coefficient of variation for this assay was 9.6%.

Paraoxonase activity and protein expression. Serum samples were assayed for paraoxonase (PON) 1 activity using paraoxon as a substrate as previously described (14). The coefficient of variation for this assay is well below 10%. PON1 and PON3 protein in serum were determined as previously described (31).

Platelet activating factor acetylhydrolase activity. Platelet-activating factor acetylhydrolase (PAF-AH) activity was measured by the method of Stafforini et al. (35). Briefly, serum samples were incubated with 2-[acetyl-³H]PAF for 30 min at 37°C. The reaction was terminated with the addition of acetic acid and [³H]acetate generated from PAF-AH activity was separated from labeled substrate by solid-phase chromatography and quantified by liquid scintillation. For this study, the coefficient of variation for this assay was 9.6%.

Statistical analysis. Statistical analyses were performed with Graph Pad Prism (GraphPad, San Diego, CA). Preintervention and postintervention values were compared using matched-pair *t*-tests. All data are expressed as means \pm SD unless otherwise indicated. A *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

Anthropometry, fasting lipids, and lipid hydroperoxides. Anthropometric and lipid data are presented in Table 1. The 21-day diet and exercise intervention significantly reduced body weight and BMI ($P < 0.01$), although as a group, the subjects remained obese (BMI >30 kg/m²) at the end of the intervention. Additionally noted were significant reductions in total cholesterol (total-C), LDL-C, TG, LDL-C-to-HDL-C ratio ($P < 0.01$ for all), and both HDL-C and total-C-to-HDL-C ($P < 0.05$ for both). No significant change in apoA-I was noted and the ratio of apoA-I to HDL increased 8%; however, this did not achieve statistical significance ($P = 0.16$). A significant

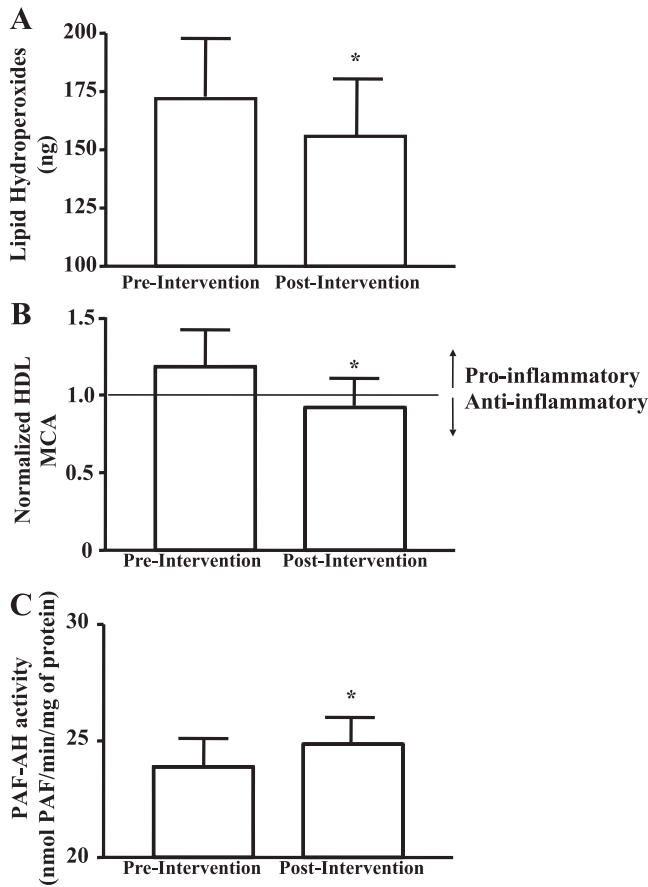


Fig. 1. Effect of diet and exercise intervention on lipid hydroperoxides (A) (**P* = 0.04 vs. preintervention), high-density lipoprotein (HDL) chemotaxis [monocyte chemoattractant activity (MCA)] (B) (**P* = 0.03 vs. preintervention), and platelet-activating factor-acetylhydrolase (PAF-AH) activity (C) (**P* = 0.05 vs. preintervention). Values are means (SD).

reduction in serum lipid hydroperoxides (155.8 ± 9.3 vs. 171.9 ± 10.8 ng; $P < 0.05$; Fig. 1A) was observed.

Inflammatory/anti-inflammatory properties of HDL. The ability of HDL to protect against MCA was tested by adding pre- and postintervention purified subject HDL to a human vascular wall coculture. Supernatants from cocultures incubated with postintervention HDL induced significantly less MCA ($P < 0.05$), and HDL was modified from proinflammatory preintervention (1.14 ± 0.11) to anti-inflammatory postintervention (0.94 ± 0.09 , $P = 0.03$, Fig. 1B). PAF-AH activity mildly increased in the post-intervention serum compared with the preintervention serum (24.6 ± 0.6 vs. 23.4 ± 0.6 nmol PAF·min⁻¹·mg⁻¹ of protein; $P = 0.05$; Fig. 1C). Serum was assayed for PON1 activity using paraoxon as a substrate. PON1 activity per milligram of HDL did not change significantly after the intervention (684.8 ± 99.7 vs. 669.2 ± 95.6 units/mg of HDL). Western blotting was used to quantify the protein levels of both PON1 and PON3. No change was noted in the protein abundance of either of these enzymes after the intervention (data not shown).

DISCUSSION

Our laboratory's prior work (4, 33, 34) established that interventions incorporating diets low in total and saturated fat

result in lower quantitative levels of HDL and provided the foundation for the present study. Given that the protective capacity of HDL against LDL oxidation may be more important than its quantitative level (20), this led us to the question of whether there is a qualitative change in the HDL inflammatory index that occurs independent of quantitative changes in HDL-C with an intervention that incorporates a high-fiber, low-fat diet. In their most recent guidelines the NCEP recommended raising HDL-C to prevent CAD (9). This recommendation may not be appropriate if subject HDL is proinflammatory, because oxidized LDL may increase susceptibility for vulnerable plaque rupture during an acute phase response. Men in the present study tended to have proinflammatory HDL on entry, suggesting their HDL, despite being quantitatively normal, was not protective against LDL oxidation. After the 3-wk diet and exercise intervention, a significant decline in the HDL inflammatory index, as measured by the ability of HDL to prevent MCA, was noted, and HDL converted from pro- to anti-inflammatory. Additionally, serum PAF-AH mildly increased and apoA-1 level was maintained despite a 10% quantitative reduction in HDL-C.

Our findings have several implications. The first issue is the quantitative importance of HDL-C. At a population level, the concentration of HDL-C is considered among the best predictors of risk, being inversely related to CAD (2). Data from Framingham indicated that >40% of events occurred in patients with normal HDL-C levels (7). The Air Force/Texas Coronary Atherosclerosis Prevention Study noted that event rate was inversely related to HDL-C level; however, event rate in those with normal HDL-C was approximately two-thirds that of the subjects with the lowest levels of HDL-C (8). More recently, Otvos et al. (29) noted that HDL-C particle number but not particle size was a predictor of CAD events. Consequently, it has been suggested that decreases in HDL-C noted with high-fiber, low-fat diets may be detrimental. The quantitative drop in HDL-C in the present study is consistent with earlier reports using the same intervention (4, 5) as well as a report by Brinton et al. (6). However, the decrease in HDL-C was coupled with larger reductions in both LDL-C and total-C, resulting in reductions in the total-C-to-HDL-C and LDL-C-to-HDL-C ratios, which also predict risk.

Second, it is now clear that in addition to lipoprotein quantity, their atherogenic properties (HDL particle size, number and inflammatory/anti-inflammatory properties, LDL particle size, number and susceptibility to oxidation) may be critical in determining atherogenic risk. For example, HDL, which can prevent LDL-induced monocyte transmigration (25), is proinflammatory during an acute phase response independent of the level of HDL (24, 26, 40). Hence, proinflammatory HDL may be a useful marker of susceptibility to atherosclerosis. To our knowledge, this is the first lifestyle investigation to assess anti-inflammatory properties of HDL, and we noted a significant increase in the HDL anti-inflammatory index (20% increase/unit HDL) despite a quantitative reduction (10%) in HDL-C, suggesting that the function of HDL-C is modified by lifestyle change independent of HDL-C levels per se. These data corroborate serum reductions in C-reactive protein that our laboratory previously noted in these subjects (34). The reduced MCA, may be explained, in part, by the decrease in monocyte chemoattractant protein-1 production by cocultures incubated with subject sera from these subjects in vitro, as



previously reported (34). It is reasonable to state that the turnover and function of HDL may be more important than the steady-state plasma levels. In a situation where low levels of HDL are formed and cleared, normal plasma levels may be misleading as to the existence of satisfactory HDL metabolism. On the contrary, under a condition where the plasma levels are low, whereas the formation and clearance of HDL are normal, the organism likely benefits from satisfactory HDL function. Brinton et al. (6) observed that the clearance of HDL increases on a low-fat diet and thus an increase in HDL turnover may facilitate the removal of proinflammatory HDL. Additionally, individuals with apoA-I(Milano) (12), despite markedly low plasma HDL-C levels, show no severe CAD. Furthermore, in a study of 27 patients with normal lipid levels, who did not have diabetes, did not smoke, were not taking hypolipidemic medications, yet had angiographically documented coronary atherosclerosis, Navab et al. (21, 23) studied the ability of patients HDL to inhibit LDL oxidation, and observed that HDL from patients was not protective against LDL oxidation. More recently, they went on to document the same effect in patients with very high HDL-C (mean HDL-C 95 mg/dl) (1) and suggested that the anti-inflammatory properties of HDL better distinguished controls from CAD patients. Although at a population level higher plasma HDL-C levels are associated with lower risk for coronary atherosclerosis, at an individual level the HDL function may be more important than plasma HDL levels.

The third issue is the mechanism for the improvement noted, which is currently unknown (20). To investigate potential contributors to the reduction in MCA, we measured the activity and/or content of four proteins associated with HDL (apoA-I, PAF-AH, PON1, PON3). PAF-AH, secreted by macrophages and associated with LDL and HDL, hydrolyzes PAF and oxidized fragments of phospholipids that are potent mediators of inflammation (36, 38). Although not universally noted (45), PAF-AH is considered to exhibit antiatherogenic properties (28). Van Lenten et al. (40) noted that enrichment of human acute-phase HDL with purified PAF-AH rendered HDL protective against LDL oxidation. We found a modest increase in serum PAF-AH after the intervention and the observed increase in PAF-AH activity may contribute, in part, to the antioxidant and anti-inflammatory benefits of the intervention.

PON1 and PON3 are HDL-associated enzymes that prevent oxidative modification of LDL and monocyte-endothelial interactions (30, 42). We found no changes in PON1 activity or PON1 and PON3 protein content. Although PON1 and PON3 did not change, the large reduction in LDL and lipid hydroperoxides suggests that the existing PON activity may better protect against LDL oxidation. We speculate that the protective capacity of HDL noted in this study is not due to PON activity because PON has been shown to vary inversely with vegetable intake (17), possibly due to the presence of increased dietary antioxidants.

ApoA-I can prevent the formation of lipid hydroperoxides from LDL-derived oxidized lipids or remove them in vivo in humans (22). It is possible that the functional ability of apoA-I was improved by this intervention. In support of this contention, it has been noted that during an acute-phase response, serum amyloid A, which is reduced by this intervention (44), displaces apoA-I in HDL. Zheng et al. (46) noted that apoA-I in human atheroma and serum is a selective target for my-

eloperoxidase-catalyzed nitration. The oxidative changes in apoA-I could account for some of the pro-inflammatory properties noted previously (11). Our laboratory noted a reduction in serum MPO in these subjects (34), and thus it is possible that apoA-I nitration may be altered by this intervention and may provide a mechanism linking lifestyle modification to decreased oxidative stress, inflammation, and dysfunctional HDL (27). Other proteins in the HDL particle, such as ApoJ or ceruloplasmin also appear to be anti-inflammatory and may have contributed to the anti-inflammatory capacity of HDL (41); however, currently there are no commercially available assays to test this.

The present study has important strengths and limitations to be acknowledged. The major strength is the supervised nature of the study. Supervising food intake and physical activity removes the need to question compliance or to rely on food intake and activity questionnaires. Although daily activity patterns were not measured, all exercise sessions were supervised and adherence to the diet and activities was essentially 100%. Despite the mixed nature of the subjects, which may have limited the ability to detect differences in some variables, significant differences were noted. We did not investigate the independent effects of diet and physical activity, and thus we cannot discern which aspect(s) of the intervention were responsible for the changes noted. However, we designed the study to investigate the combined effect of diet and physical activity, as both are recommended for optimal prevention of CAD (9). Additionally, because the subjects had exercised the day before the final blood draw, we cannot separate out the chronic vs. acute effects, although many of the variables reported in prior studies are not impacted by an acute bout of exercise, whereas others are influenced for a short time frame postexercise. Interestingly, some have reported that a strenuous bout of exercise increases proinflammatory status (39).

In conclusion, multiple factors can modulate inflammation associated with atherosclerotic lesions, including HDL. The reduced MCA observed using the subject sera after the intervention is a clear indication of improved anti-inflammatory properties of HDL and likely contributed to a reduction in the "chronic acute phase response" (20) that existed in these overweight/obese subjects with metabolic syndrome factors. These observations suggest that despite a quantitative reduction in HDL, appropriate diet and exercise may confer risk reduction resulting from turnover of pro-inflammatory HDL. Future research should investigate these effects of HDL anti-inflammatory properties with regard to the number and size of HDL particles, the oxidative status of apoA-I, the independent effects of diet and exercise, and the relation to CAD events.

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