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Obesity favors apolipoprotein E- and C-III-containing high density lipoprotein subfractions associated with risk of heart disease.

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Abstract  Human HDLs have highly heterogeneous composition. Plasma concentrations of HDL with apoC-III and of apoE in HDL predict higher incidence of coronary heart disease (CHD). The concentrations of HDL-apoA-I containing apoE, apoC-III, or both and their distribution across HDL sizes are unknown. We studied 20 normal weight and 20 obese subjects matched by age, gender, and race. Plasma HDL was separated by sequential immunoaffinity chromatography (anti-apoA-I, anti-apoC-III, anti-apoE), followed by nondenaturing-gel electrophoresis. Mean HDL-cholesterol concentrations in normal weight and obese subjects were 65 and 50 mg/dl \( (P = 0.009) \), and total apoA-I concentrations were 119 and 118 mg/dl, respectively. HDL without apoE or apoC-III was the most prevalent HDL type representing 89% of apoA-I concentration in normal weight and 77% in obese \( (P = 0.01) \) individuals; HDL with apoE-only was 5% versus 8% \( (P = 0.1) \); HDL with apoC-III only was 4% versus 10% \( (P = 0.009) \); and HDL with apoE and apoC-III was 1.5% versus 4.6% \( (P = 0.004) \). Concentrations of apoE and apoC-III in HDL were 1.5–2× higher in obese subjects \( (P \approx 0.004) \). HDL with apoE or apoC-III occurred in all sizes among groups. Obese subjects had higher prevalence of HDL containing apoE or apoC-III, subfractions associated with CHD, whereas normal weight subjects had higher prevalence of HDL without apoE or apoC-III, subfractions with protective association against CHD.

Epidemiological studies have shown that low plasma levels of HDL cholesterol (HDL-C) are strongly associated with an elevated risk of coronary heart disease (CHD) \( (1–5) \). However, evidence from randomized clinical trials that studied drugs that increased HDL-C has not been consistent with the hypothesis that HDL protects against CHD. In large-scale trials of novel cholesteryl ester transfer protein inhibitors, CHD incidence was not reduced despite substantial increases in HDL-C levels \( (6–8) \); trials of estrogen replacement therapy in postmenopausal women did not confirm a protective effect on CHD despite increases in HDL-C \( (9–11) \); and two recent trials of niacin also failed to show reduction in CHD risk, although the increases in HDL-C were modest \( (12, 13) \). Furthermore, some genetic variation that is associated with high HDL-C concentration is not associated with reduced CHD \( (14) \). This raises questions regarding the efficacy of HDL-C elevation, in general, as a strategy for CHD prevention.

Accumulating evidence indicates that protein composition of HDL may be relevant to the risk of CHD \( (15–17) \). ApoE and apoC-III are found on the surface of both triglyceride-rich lipoproteins (TRLs) and HDL \( (18–20) \). The concentration of apoE in HDL is an independent predictor of recurrent coronary events \( (15) \). More recently, a proteomic analysis showed greater apoE enrichment in small-size HDL \( (HDL_3) \) in subjects with established coronary artery disease than in normal controls \( (16) \). Similarly, HDL containing apoC-III independently predicts increased risk of an initial coronary event in separate cohorts of men and women \( (17) \), and a high ratio of apoC-III to apoA-I in HDL predicts recurrent coronary events \( (15) \).

**Supplementary key words** apolipoprotein A-I • coronary heart disease • high density lipoprotein size
Distinct HDL speciation based on apoC-III and apoE content is not firmly established in the literature. ApoC-III and apoE long have been known to be present in HDL (19, 20). Findings in proteomics studies showed that apoC-III and apoE are present throughout the range of sizes of HDL that are prepared by ultracentrifugation or gel filtration, or by precipitation of apoB lipoproteins (21–23), and analysis of HDL apolipoprotein correlations suggests speciation. However, it is not settled to what extent apoC-III and apoE define distinct subtypes of apoA-I-containing HDL as they do on the apoB lipoproteins (18, 19, 24); whether apoC-III and apoE coexist on HDL as they do on VLDL and LDL (18, 19, 24, 25); and how they are distributed among smaller- or larger-size apoA-I-containing HDL particles. One study, using immunoadfinity separation, showed that apoE is present in apoA-I-containing lipoproteins in small and large sizes (26). Even so, there remains the issue of conflicting findings involving previous studies proposing that most if not all apoE in HDL is found on particles that do not have apoA-I as suggested by non-overlapping regions of apoE and apoA-I on nondenaturing two-dimensional gel electrophoresis with immunoblotting (27, 28) or with reconstituted HDL particles (29). In contrast, other studies using anti-apoA-I immunoafi nity or heparin binding demonstrated that at least a portion of HDL has both apoE and apoA-I (19, 26, 30–33). Much less research has been done on apoC-III as a component of apoA-I HDL.

The purpose of this study was to examine apoA-I-containing HDL subpopulations as defined by apoE and apoC-III content as well as by size in the context of normal body weight and obesity, a clinical condition related to CHD. We studied the distribution of these different apolipoprotein-defined HDL types in a group with normal HDL-C levels and normal body weight and compared it with that of an obese group, a common high-risk phenotype associated with low HDL-C. Elevated apoC-III concentrations in HDL (34, 35), as well as increased apoC-III production (36), are common features of obesity. We hypothesized that the distribution of HDL particles is disturbed with obesity, such that obese subjects have higher concentrations of HDL with apoC-III as well as HDL with apoE, and lower concentrations of HDL without apoE or apoC-III contributing to their increased risk for CHD.

METHODS

Study population

The study population consisted of 40 participants, 26 women and 14 men, age 30–67 years, assigned to two different groups based on BMI. The obese group (n = 20) was defined as having a BMI $\geq$30, while the normal weight group (n = 20) was defined as having a BMI $\leq$25. Subjects for the obese group were selected from the POUNDS LOST study, a randomized clinical trial comparing diets for weight loss (37). Briefly, the eligibility criteria for the parent study were age 30–70 years and BMI between 25 kg/m$^2$ and $\leq$40 kg/m$^2$. Major exclusions in the parent study were diabetes, unstable cardiovascular disease, and use of lipid-lowering medications or medications that affect body weight. Participants selected from the POUNDS LOST study for our obese group had a BMI $\geq$30, and the samples used were baseline samples, prior to dietary intervention. Participants for the normal weight group were healthy volunteers with a BMI $\leq$25 and were selected using the same major exclusions as for the obese group. Obese subjects were matched to normal weight subjects by age, gender, and race. We aimed to study a representative group of matched normal and obese participants that would have more generalizability than a select population subgroup. All study participants gave informed consent, and the Human Subjects Committees of the Harvard School of Public Health and Brigham and Women’s Hospital approved the study.

Blood collection, storage, and measurements

Blood was collected from participants following at least an 8 h fast. Serum was separated and aliquotted by trained personnel at each clinical site and stored at $-80^\circ$C. Vials containing 0.5 ml of frozen plasma were sent from Pennington Biomedical Research Center to the lipoprotein laboratory at the Harvard School of Public Health. Samples from both study groups were collected and stored in frozen vials for an average of 2 years before analysis of lipoprotein types was conducted, with the exception of five samples from our normal weight study group that were collected and stored in frozen vials 6 months before analysis. The laboratory personnel were blinded to the samples’ group status.

Isolation of HDL from plasma by immunoadfinity chromatography

Plasma samples were removed from cryogenic storage, thawed, and filtered for the removal of fibrinogen and coagulation products using Pall (R) Acrodisc filters (5 µm). ApoA-I-containing lipoproteins were then separated from plasma using immunoaffinity-purified polyclonal anti-apoA-I antibodies as follows (Fig. 1): 1 ml of filtered plasma was loaded and incubated in 20 ml Econo-Pac columns (Bio-Rad Laboratories, Hercules, CA) packed with 2.5 ml of affinity-purified polyclonal goat anti-human apoA-I antibodies (Academy Bio-Medical Co., Houston, TX) bound to Sepharose 4B resin. The unbound lipoproteins were collected by gravity flow followed by washes with PBS and stored at $-80^\circ$C. The bound fraction, which was the fraction of interest, was then eluted from the columns with 3M sodium thiocyanate (NaSCN) in PBS and immediately desalted by multiple rinses in Vivaspin 20 ultrafiltration centrifugal device with polyethersulfone (PES) membrane at 10,000 Da molecular weight cutoff (MWCO) (Sartorius Stedim Biotech, Germany), ending with a final sample volume of 500 µl. This apoA-I-containing fraction was then loaded sequentially onto anti-apoC-III columns followed by anti-apoE columns for further separation of apoA-I-containing lipoproteins by apoE and apoC-III content (18, 38). In detail, the apoA-I-containing fraction was loaded and incubated under similar conditions onto columns packed with affinity-purified polyclonal antibodies anti-apoC-III (DMA, Arlington, TX). The unbound fractions (C-III) were collected by gravity flow from the columns, and the resin was washed with PBS. The bound fraction (C-III) was eluted by incubation with 3M NaSCN in PBS and immediately desalted by multiple rinses as described for the apoA-I-containing fraction. The C-III’ fractions and the dialyzed C-III’ fractions were then finally loaded and incubated in columns packed with affinity-purified polyclonal antibodies anti-apoE (Genzyme, Cambridge, MA). The same elution protocol used for the anti-apoA-I and the anti-apoC-III resin was carried out. This yielded four distinct subfractions: HDL without apoE or apoC-III (E’CIII’), HDL with apoE but without apoC-III (E’CIII’), HDL with apoC-III but without apoE (E’CIII’), and

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We also tested the effects of plasma filtration on laboratory controls by measuring the total whole plasma apoA-I concentration, in both filtered and unfiltered plasma samples, and found no significant difference (mean of 124 ± 21 mg/dl for the unfiltered vs. 114 ± 10 mg/dl for the filtered, \( P = 0.1 \)), concluding that any precipitation of lipoproteins that may or may not be occurring with plasma thawing is not removed by filtration. In addition, we tested whether any apoC-III is lost through the 10,000 Da MWCO filter as a result of apolipoprotein dissociation during the immunoaffinity chromatography steps. We separated plasma controls by anti-apoA-I columns and loaded the apoA-I-containing fraction onto anti-apoC-III columns. The eluted apoA-I containing apoC-III was then concentrated with 10,000 MWCO concentrators. The filtrates that would normally be discarded after this step were analyzed with a highly sensitive ELISA. Measurement of apoC-III was undetectable. Therefore, we conclude that apoC-III remains with the HDL particle during the preparation steps. Finally, this experiment shows that even a very small intact HDL, having a single apoA-I at 28,800 Da with some lipid and apoC-III, would not be lost through the 10,000 Da MWCO filter.

Because it has been shown that apoA-I is present in LDL, we measured the apoB concentration in the elution obtained from anti-apoA-I immunoaffinity chromatography of plasma samples from laboratory controls with a high-sensitivity sandwich ELISA using affinity-purified antibodies (Academy Bio-Medical Co.) and a horseradish peroxidase/ortho-phenylenediamine detection system. This ELISA has a lower limit of detection of 0.0015 mg/dl. We found that only 2.7% of whole plasma apoB was found in the apoA-I bound fraction. More so, after further separation of apoA-I-containing lipoproteins using anti-apoE and anti-apo-C-III columns, 94% of the apoB measured did not

Fig. 1. Separation of HDL as defined by apoE and apoC-III content, as well as by size. ApoA-I-containing HDL subfractions were separated from plasma samples by first using anti-apoA-I, anti-apoC-III, and anti-apoE immunoaffinity chromatography in sequence followed by size separation using nondenaturing polyacrylamide gel electrophoresis. Sixteen distinct apoA-I lipoprotein types were obtained: [very small-size (discoi dal), small-size, medium-size, and large-size HDL], E’ CIII’, E’ CIII’, E’ CIII’, and E’ CIII’.

HDL with both apoE and apoC-III (E’ CIII’). All incubations occurred overnight in the cold room with constant mixing. The efficiencies of the apoC-III and apoE immunoaffinity separation (percentage of ligand removed from plasma by the resin) were 97% and 94%, respectively. After collection of the bound fraction, the immunoaffinity columns were washed three times with NaSCN and with PBS containing EDTA. Then the columns were reconditioned with 0.1 M acetic acid and finally washed with PBS. The columns were completely clean of apoC after this procedure. For example, the third wash with NaSCN eluted <0.4% of plasma apoA-I. No apoA-I was detected after a fourth wash, showing that the washing procedure removed all the bound apoA-I.

To address a concern on the possible transfer of apoC-III from VLDL to HDL during freezing or storage (39), we measured apoC-III in VLDL, LDL, and HDL immediately after plasma separation by density gradient ultracentrifugation and after 5 months of storage at \(-80^\circ\)C in samples from four normal individuals. The distribution of apoC-III between TRLs and HDL was similar [14% in VLDL, 16% in LDL, and 70% in HDL, when analyzed fresh; 12% in VLDL, 15% in LDL, and 73% in HDL after frozen storage; multivariate ANOVA (MANOVA) \( P = 0.1 \)]. We then tested the effects of storage and thawing on apoA-I-containing HDL when separated by immunoaffinity chromatography and found no differences in apoE or apoC-III distribution on HDL between fresh and previously stored control plasma samples: mean apoA-I HDL without apoE was 135 ± 5 mg/dl and HDL with apoE 10 ± 0.7 mg/dl in the fresh samples versus 134 ± 5.6 mg/dl and 7 ± 2 mg/dl in the frozen samples (\( P = 0.5 \) and 0.1); similarly, mean apoA-I HDL without apoC-III was 137 ± 5 mg/dl and HDL with apoC-III 8.4 ± 1 mg/dl in the fresh samples versus 135 ± 4 mg/dl and 10 ± 1.5 mg/dl in the frozen samples (\( P = 0.2 \) and 0.1, respectively).

We also tested the effects of plasma filtration on laboratory controls by measuring the total whole plasma apoA-I concentration, in both filtered and unfiltered plasma samples, and found no significant difference (mean of 124 ± 21 mg/dl for the unfiltered vs. 114 ± 10 mg/dl for the filtered, \( P = 0.1 \)), concluding that any precipitation of lipoproteins that may or may not be occurring with plasma thawing is not removed by filtration. In addition, we tested whether any apoC-III is lost through the 10,000 Da MWCO filter as a result of apolipoprotein dissociation during the immunoaffinity chromatography steps. We separated plasma controls by anti-apoA-I columns and loaded the apoA-I-containing fraction onto anti-apoC-III columns. The eluted apoA-I containing apoC-III was then concentrated with 10,000 MWCO concentrators. The filtrates that would normally be discarded after this step were analyzed with a highly sensitive ELISA. Measurement of apoC-III was undetectable. Therefore, we conclude that apoC-III remains with the HDL particle during the preparation steps. Finally, this experiment shows that even a very small intact HDL, having a single apoA-I at 28,800 Da with some lipid and apoC-III, would not be lost through the 10,000 Da MWCO filter.
Separation of HDL size fractions using nondenaturing polyacrylamide gel electrophoresis

Each of the four immunofractions was then separated on a gradient gel and classified into four distinct sizes based on the nomenclature proposed by Rosenson et al. (40) (Fig. 2). First, the eluted fractions were concentrated to a volume of 50 μl using Corning Spin-X UF concentrators with PES membrane at 10,000 Da MWCO (Corning, UK) and then loaded onto a precast 4–30% polyacrylamide gradient gel (Jule Inc., Milford, CT). Molecular weight calibration standard (Amersham, GE Healthcare, UK) in the range of 66 to 669 kDa was loaded onto the first well to identify size bands of interest (41). The gel was then run in 0.1 M Tris/borate/EDTA buffer for 16 h at 70 V by using the XCell SureLock electrophoresis apparatus and EPS 500/400 power supply. Subsequently, the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane using a Hoefer semidy transfer unit at 30 V for 16 h. To visualize the HDL subpopulations, the membrane was stained with 0.2% amido-black stain for 20 min followed by four rinses with distilled water. Bands below 66 kDa were identified as very small-size (discoidal) apoA-I-containing HDL. Those ranging from 66 to 140 kDa were identified as small-size HDL; from 140 to 232 kDa, medium-size HDL; and from 232 to 440 kDa, large-size HDL. This process yielded 16 distinct HDL subfractions based on apoE and apoC-III content, as well as size fraction: [very small (discoidal), small, medium, and large HDL], E ‘CIII’, E ‘CII’, E ‘CII’, and E ‘CIII’.

Quantification of HDL subpopulations

Using PVDF elution buffer (containing 6.05 g Tris-Trizma base, 10 g Triton x100, and 20 g SDS per 1,000 ml), protein for each HDL subfraction was eluted from the membrane and then concentrated to a volume of 20 μl in Corning Spin-X UF concentrators with PES membrane at 10,000 MWCO. Five microliters of DTT was added to the sample as a reducing agent (NuPAGE, Invitrogen, Carlsbad, CA), along with 25 μl of SDS sample buffer, and the final volume was loaded onto a precast 4–20% Tris-Glycine gel (Novex, Invitrogen) for denaturing electrophoresis. Molecular weight calibration standard (SeeBlue Prestained, Invitrogen) in the range of 4 kDa to 250 kDa was loaded onto the first well to identify a band of interest at ~28 kDa, the molecular mass for apoA-I. In addition, human apoA-I at a concentration of 1 mg/ml (Academy Bio-Medical Co.) was used as a second standard to verify the location of all apoA-I-containing HDL bands on the gel. Electrophoresis was run in Tris-Glycine SDS buffer for 2 h at 125 V using XCell SureLock electrophoresis apparatus and EPS 500/400 power supply. Gels were then washed with distilled water, stained with Coomassie G-250 blue stain (SimplyBlue SafeStain, Invitrogen) for band visualization, and photographed with an InGenius transilluminator imaging system (Syngene, Synoptics Group, UK) (Fig. 3). HDL subpopulation distribution was estimated by quantifying individual band intensity using GeneTools image analysis software (Syngene, Synoptics Group) and applying the percent distribution to the total apoA-I concentration. Recovery after the three sequential immunoadfinity separations averaged 34%.

To confirm the nature of the 60 kDa bands observed in our last step involving quantification of apoA-I-containing HDL subpopulations on the denaturing gel (Fig. 3), we transferred the gel onto a PVDF membrane and performed Western blotting using affinity-purified goat anti-human albumin polyclonal antibody as the primary antibody, confirming that the bands on the 60 kDa molecular mass markers are albumin and excluding the possibility of these bands representing dimerization of apo A-I, which in turn would affect the quantification of the HDL size subpopulations.

Measurement of apo levels

ELISA using affinity-purified antibodies (Academy Bio-Medical Co.) was performed to determine concentrations of apoA-I, apoC-III, and apoE in whole plasma and the four HDL immunofractions. ELISA plates were read with a BioTek ELX808iu 96-well plate reader controlled by KC Junior software (BioTek, Winooski, VT). All assays were completed in triplicate, and any sample with an intra-assay coefficient of variation >15% was repeated. Final data were exported to Microsoft Excel for analysis and database management.

ApoE genotyping

To better understand if our results could be influenced by the varied affinity of apoE for different lipoprotein classes as a result of genotype (apoE 2/3/4), we performed apoE genotyping on plasma samples using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), in 384-well format. The 5′ nucleic assay (TaqMan®) was used to distinguish the two alleles of a gene. PCR amplification was carried out on 5–20 ng DNA using 1 X TaqMan® universal PCR master mix (No
RESULTS

Subject characteristics

Participants in both groups were similar in age, gender, and race, as intended by matching (Table 1). The average age was 51 years; roughly two-thirds of the participants were women, predominantly white. Twenty-two of the 26 women were postmenopausal; 3 premenopausal women were in the normal weight group, and 1 in the obese group. The obese group had a mean BMI of 33.2 ± 3.1 kg/m² compared with the BMI of the normal weight group, 22.5 ± 1.9 kg/m². Obese participants had significantly higher fasting triglycerides, insulin levels, and HOMA index of insulin resistance. Although the average HDL-C level for the normal weight group was significantly higher compared with the obese group, 65 and 50 mg/dl, respectively (P = 0.009), the mean total plasma apoA-I concentration was similar between the groups (119 and 118 mg/dl). Total plasma apoE and apoC-III concentrations were significantly higher in the obese group compared with the normal weight group (P = 0.002 and 0.0001, respectively).
HDL types in plasma as defined by apoE and apoC-III content

Percentage distribution. E’CIII’ was the most prevalent of four apo-defined HDL types studied across groups, representing ~89% of the total apoA-I concentration in plasma in the normal weight group and 77% in the obese group (P = 0.01) (Fig. 4). Accordingly, about 11% of HDL in normal weight subjects contained apoE, apoC-III, or both, compared with 23% in obese subjects (P = 0.01). E’CIII’ was not significantly higher in the obese than in the normal weight group, 8.3% versus 5% (P = 0.1). E’CIII’ represented 9.7% in the obese versus 4.2% in the normal weight group (P = 0.009). Finally, E’CIII’ was the least prevalent in both study groups, 4.6% in the obese and 1.5% in the normal weight group (P = 0.004). A global test for a difference in overall percent distribution of the four HDL types between the normal weight and obese groups was significant (MANOVA P = 0.01). The results were similar when restricted to the postmenopausal women (supplementary Table I).

ApoA-I concentration of HDL types

The mean apoA-I concentration of E’CIII’ was 107 mg/dl in the normal weight group, compared with 91 mg/dl in the obese group (P = 0.08) (Table 2). Mean apoA-I concentrations of E’CIII’ were 5.8 mg/dl in the

Fig. 4. HDL distribution as defined by apoE and apoC-III content. The percent of total plasma apoA-I E’CIII’, E’CIII’, E’CIII’, and E’CIII’. P values <0.05 were considered to indicate statistical significance for the difference between normal weight and obese groups as determined by MANOVA test on logit transformations of variables expressed in percentages. Overall MANOVA test for difference in the percent distribution of the four apo-defined HDL types between normal weight and obese groups is significant, P = 0.01.
normal weight group and 9.8 mg/dl in the obese group \( (P = 0.1) \); of E’ CIII was 5.2 mg/dl and 11.4 mg/dl \( (P = 0.01) \); and of E’ CIII’ were 1.8 mg/dl and 5.4 mg/dl, respectively \( (P = 0.004) \) (Table 2). All together, the plasma concentrations of apoA-I-containing apoA-I lipoproteins were >2-fold higher in the obese compared with the normal weight group, 15.2 mg/dl versus 7.6 mg/dl \( (P = 0.02) \). Similarly, the obese group had ~2.5-fold higher apoA-I concentrations of apoC-III-containing HDL than the normal weight group, 16.8 mg/dl versus 7 mg/dl \( (P = 0.006) \). A global test for a difference in overall apoA-I concentration distributed across the four HDL types between normal weight and obese groups was significant (MANOVA \( P = 0.03) \).

Distribution of HDL types within very small, small, medium, and large sizes

The distribution of HDL sizes was significantly different in the obese compared with the normal weight group (MANOVA \( P = 0.007) \) (Table 3). The obese group had more very small-size (discoidal) HDL \( (17 \text{ mg/dl vs. } 12 \text{ mg/dl}) \) and less large-size HDL \( (17 \text{ mg/dl vs. } 25 \text{ mg/dl}) \) than the normal weight group. The intermediate sizes, small and medium, together were similar in the two groups, 82-83 mg/dl. In this size range, medium-size HDL was significantly higher in the obese compared with the normal weight group, 32 versus 22 mg/dl \( (P = 0.03) \).

HDL types in plasma as defined by apoE and apoC-III content and size

Comparison of plasma apoA-I concentrations across 16 HDL subfractions as defined by apoE and apoC-III content, as well as particle size, showed that the presence of apoE and/or apoC-III was not exclusive to any one specific HDL particle size (Table 4); that is, apoE and apoC-III were present in all sizes of HDL in both normal weight and obese subjects. Overall MANOVA test for difference in apoA-I concentration of the 16 HDL subfractions as defined by apoE and apoC-III content and size between normal weight and obese groups was significant, \( P = 0.01) \) (Table 4). The obese group had a higher apoA-I concentration of the two types of apoC-III-containing HDL \( (E’ CIII’, E’ CIII’) \) across most size fractions. The concentrations of apoE-containing HDL \( (E’ CIII’, E’ CIII’ \) were also higher across most size fractions in the obese group except for large-size HDL \( E’ CIII’ \), which was lower than the normal weight group \( (P = 0.04) \).

Concentrations of apoE and apoC-III in HDL

The concentration of apoE in HDL was ~60% higher in the obese group, 6.5 ± 3.3 mg/dl compared with 4.1 ± 1.0 mg/dl in the normal weight group \( (P = 0.004) \) (Table 2). Similarly, the concentration of apoC-III in HDL was 90% higher in the obese group, 11.9 ± 4.5 mg/dl versus 6.7 ± 3.2 mg/dl \( (P = 0.0001) \). Calculated molar ratios of apoE:apoA-I and apoC-III:apoA-I were not significantly different between study groups, with an average of about one apoE molecule per apoA-I in apoE-containing HDL particles, and four apoC-III molecules per apoA-I in apoC-III-containing HDL.

ApoE genotyping was studied in all subjects (with the exception of two normal weight individuals, whose DNA samples were unavailable for analysis). The vast majority of individuals from both normal weight and obese groups shared the same apoE genotype \( (E3/E3 \text{ homozygote}) \), and none of the subjects from either group were E2/E2 or E4/E4 homozygotes, suggesting that differences in the distribution of apoE on the 16 apoA-I-HDL subpopulations characterized in our study is not likely influenced by differences in apoE genotype between groups (Table 1).

### DISCUSSION

We compared in obese and normal weight participants the concentration and relative distribution of four distinct apoA-I HDL types present in plasma as defined by their
apoE and apoC-III content, and across sizes including very small (presumably discoidal) HDL and small, medium, and large HDL. Our central finding is a higher prevalence of HDL without apoE or apoC-III in normal weight participants and a higher prevalence of apoC-III-containing HDL, with or without apoE, in the obese individuals. Because HDL with apoE or apoC-III is associated with CHD (15–17), this suggests that obese people have a type of HDL that does not protect them against cardiovascular disease as well as the HDL in those with normal body weight.

Only ~6–7% of apoA-I was associated with apoC-III or apoE in the normal weight group, and about double that in the obese group. HDL apoC-III directly predicts CHD (15–17). HDL apoC-III lacks a protective property of total HDL, reduction of monocyte adhesion to vascular endothelial cells (42), which is an early step in atherogenesis. The apoE concentration in HDL is also a direct predictor of coronary events (15) and was found to be more prevalent in small-size HDL in a small group of subjects with established coronary artery disease when compared with controls (16).

A relatively high content of apoE in HDL could adversely affect its function. Consistent with previous studies, we found that the distribution of HDL sizes was significantly shifted from large to small in obesity (43–48). This shift involved a lower prevalence of large-size HDL with apoE and a higher prevalence of small-size HDL with apoE in the obese group. Supporting this finding, some studies in subjects with established coronary artery disease and with low HDL-C concentration have found lower apoE levels in the larger HDL2 (49) and higher apoE levels in smaller HDL3 (16). ApoE may serve as a ligand for hepatic LDL receptors facilitating HDL clearance from plasma (50, 51). It is possible that increased concentrations of apoE in HDL drive premature apoE-mediated clearance of HDL particles contributing to lower HDL-C levels in the obese. This suggests the possibility that redistribution of apoE from large to small HDL particles impairs reverse cholesterol transport from peripheral tissues to the liver, and this might be one of the underlying mechanisms for increased CHD risk in obese with observed lower concentrations of large apoE-enriched HDL. Thus, the increased presence of apoC-III and apoE on HDL particles may contribute to the formation of less protective or dysfunctional HDL and may represent one of the mechanisms for the

**Table 3.** Plasma apoA-I concentrations in mg/dl across HDL subfractions as defined by size

<table>
<thead>
<tr>
<th>Normal Weight (n = 20)</th>
<th>Obese (n = 20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very small 12 ± 7</td>
<td>17 ± 11</td>
<td>0.1</td>
</tr>
<tr>
<td>Small 60 ± 30</td>
<td>51 ± 15</td>
<td>0.1</td>
</tr>
<tr>
<td>Medium 22 ± 14</td>
<td>32 ± 18</td>
<td>0.03</td>
</tr>
<tr>
<td>Large 25 ± 18</td>
<td>17 ± 11</td>
<td>0.1</td>
</tr>
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</table>

Means ± SD. Very small (discoidal) HDL, 66 kDa or smaller; small HDL, 66–140 kDa; medium HDL, 140–232 kDa; large HDL, 232–440 kDa. P values <0.05 were considered to indicate statistical significance for the difference between normal weight and obese groups as determined by MANOVA test of variables expressed as means ± SD. Overall MANOVA test for difference in apoA-I concentration of the four HDL sizes between normal weight and obese groups is significant, P = 0.007.

**Table 4.** Plasma apoA-I concentrations and relative distribution across 16 distinct HDL subfractions as defined by apoE and apoC-III content, as well as size

<table>
<thead>
<tr>
<th>Normal Weight (n = 20)</th>
<th>Obese (n = 20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E' CIII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very small 9.9 ± 7.4</td>
<td>12.9 ± 10.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Small 55.4 ± 29.0</td>
<td>38.0 ± 13.9</td>
<td>0.009</td>
</tr>
<tr>
<td>Medium 18.9 ± 13.7</td>
<td>25.7 ± 17.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Large 22.3 ± 17.4</td>
<td>14.1 ± 10.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Subtotal 107 ± 28</td>
<td>91 ± 28</td>
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</tbody>
</table>

| E' CIII                 |                |   |
| Very small 1.1 ± 0.9    | 1.4 ± 1.3      | 0.7 |
| Small 1.8 ± 1.2         | 5.4 ± 5.2      | 0.2 |
| Medium 1.2 ± 1.0        | 2.1 ± 3.2      | 0.7 |
| Large 1.7 ± 1.6         | 0.9 ± 0.8      | 0.04 |
| Subtotal 5.8 ± 4.0      | 9.8 ± 9.7      | 100% |

| E' CIII                 |                |   |
| Very small 0.7 ± 0.5    | 1.4 ± 1.0      | 0.02 |
| Small 2.7 ± 2.6         | 5.8 ± 4.1      | 0.02 |
| Medium 1.1 ± 1.0        | 2.9 ± 3.2      | 0.02 |
| Large 0.7 ± 0.6         | 1.3 ± 1.2      | 0.1 |
| Subtotal 5.2 ± 4.3      | 11.4 ± 10.2    | 100% |

Means ± SD. P values <0.05 were considered to indicate statistical significance for the difference between normal weight and obese groups as determined by MANOVA test of variables expressed as mean ± SD. Overall MANOVA test for difference in apoA-I concentration of the 16 HDL subfractions as defined by apoE and apoC-III content and size between normal weight and obese groups is significant, P = 0.01.
increased risk of CHD associated with low HDL-C in the obese.

We found that a small percentage (23%) of apoE-containing HDL also contained apoC-III in the normal group, which increased to 36% in the obese group. Likewise, only 26% of apoC-III-containing HDL also contained apoE in the normal weight group and 32% in the obese group. Therefore, HDLs with apoC-III or apoE are mostly distinct from each other. Nonetheless, colocalization of apoE and apoC-III in HDL should be considered in studies in CHD risk prediction concerning either HDL apolipoprotein. For example, one could modify or confound a potential relation of the other to cardiovascular disease.

This study supports and extends previous studies that showed that apoE is present in apoA-I HDL (19, 26, 30–33). Weisgraber et al. (30) found that HDL, prepared by ultracentrifugation, contained subfractions that did or did not contain apoE, demonstrated by heparin binding. Both HDL types were rich in apoA-I. Alaupovic and colleagues (33), using anti-apoA-I immunoaffinity chromatographic separation, found that 50% to 75% of plasma apoE and apoCs are present in apoA-I-containing lipoproteins in 16 normal children and 15 normal adults. Several additional studies found that apoE is associated with apoA-I lipoproteins prepared by immunoaffinity (26, 31–33). Hannuksela et al. (31) and Castro and Fielding (32), in addition to finding apoE and apoA-I together in HDL, found that some apoE-containing HDL did not have apoA-I. In fact, Castro and Fielding demonstrated that apoE was present in HDL that did not contain apoA-I only when HDL was prepared by ultracentrifugation or polyanionic precipitation. They found that these two methods produced these HDLs with apoE only by dissociating apoE from apoB lipoproteins. In contrast, Krimbou et al. (28) and Asztalos et al. (27) subfractionated HDL directly from plasma by sequential agarose and polyacrylamide electrophoresis followed by anti-apoA-I and anti-apoE immunoblotting. ApoA-I and apoE were located mostly in separate areas of the membrane. Thus, the existence of HDL that has apoE but not apoA-I may be dependent on the method of preparing HDL.

It is possible that higher apoC-III and apoE levels in the TRLs of obese participants enrich HDL with these apos by exchange during circulation in plasma. ApoC-III could slow the clearance of HDL from the circulation, as it does in VLDL and LDL, leading to higher circulating concentrations of HDL with apoC-III. It is also possible that other apos could reside on HDL with apoC-III or HDL with apoE that prolong their circulation.

The groups in our study were carefully matched for age, gender, and race, in recognition of the known variability of HDL between these groups. One limitation to our study is that we did not evaluate the effects of body weight change on HDL. Obesity was studied in a cross-sectional design, so causality for obesity and prevalence of the apoE- and apoC-III-containing HDL types cannot be assumed. It is possible that the difference in apoC-III HDL concentrations observed between our groups could be a function of hypertriglyceridemia and insulin resistance (36) in the setting of obesity. More so, isolated plasma apo measurements can offer only a snapshot of HDL at a particular moment in time, while metabolism studies in HDL would be more informative about HDL synthesis, intravascular remodeling, and catabolism.

In conjunction with low HDL-C levels, the obese participants had higher fasting triglycerides and elevated markers of insulin resistance, representing the typical low-HDL phenotype that is associated with high risk of CHD, as in the metabolic syndrome. We intended to characterize the apoE- and apoC-III-defined HDL types in this common phenotype to gain understanding of a low-HDL phenotype associated with high risk, rather than unusual low-HDL phenotypes caused by genetic variation such as LCAT deficiency, apoA-I gene mutation, or Tangier’s disease. The results of this study extend the abnormal HDL phenotype of low plasma levels and small size of HDL-C to HDL types based on proteins that are known to have functions on HDL that affect its interaction with vascular cells and perhaps clearance rates from plasma. The obese group had total apoA-I levels that were similar to the normal weight group; nonetheless, their apoA-I was distributed much more to apoE- and apoC-III-containing fractions. ApoE and apoC-III may be more specific markers for abnormal HDL than low plasma concentrations of total apoA-I or HDL-C. These findings may have therapeutic implications when evaluating lifestyle or pharmacological interventions, aimed at lowering CHD risk, as HDL containing these apos may represent a superior target for treatment. Cardioprotective benefits may depend on the ability to affect the concentrations of protective versus nonprotective HDL types.

REFERENCES


