SUPPLEMENTAL MATERIAL: HDL subspecies defined by apolipoprotein C-III and incident coronary heart disease in four cohorts

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METHODS

Danish Register Linkage

Cases with a first-time discharge diagnosis of MI (ICD 8: 410-410.99; ICD 10: I21.0-I21.9) were identified in the National Register of Patients, and sudden cardiac death cases (ICD 8: 427.27 or ICD 10: I46.0-I46.9) were identified from the Cause of Death Register if the cardiac arrest after verification of death certificate was believed to be caused by an MI (Joensen et al. J Clin Epidemiol 2009).

Case-control studies nested within the NHS and HPFS

As described in our paper in 2012 (Jensen et al. JAHA 2012), nested case-control studies were designed within the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS), allowing the study to maintain a prospective design. A total of 286 incident cases were identified in NHS and 348 in HPFS and controls were matched 1:1 on age (1 year), smoking (never, past, current), and month of blood return, among participants who were free of cardiovascular disease at the time CHD was diagnosed in the case (risk set sampling). The diagnosis of CHD included non-fatal MI and fatal CHD.

Assessment of HDL subtypes according to apoC-III

The methodology for assessing HDL with and without apoC-III has developed over time. In the initial paper for the Nurses' Health Study and the Health Professionals' Follow-Up Study (Jensen et al. JAHA 2012), plasma was separated according to apoC-III by immuno-affinity chromatography and HDL-cholesterol was measured after ultracentrifugation to remove apoB-lipoproteins. Because ultracentrifugation has some limitations in terms of efficiency as well as the potential changes in HDL composition, we switched to measurement of apoA-I and HDL-

cholesterol concentrations in apoB-depleted samples in the Diet, Cancer and Health Study (described in detail below). We used the opportunity to compare results using apoA-I and HDL-cholesterol as surrogate measures of HDL, and found similar results when using apoA-I or HDL-cholesterol with and without apoC-III as our exposure. Thus, we focused on apoA-I with and without apoC-III for the MESA study as well (details below).

Laboratory methods: Diet, Cancer and Health Study

In the DCH samples, triglycerides were assessed in total plasma by enzymatic assay (Thermo Fisher Scientific, Waltham, MA) and total apoB concentrations were calculated as the sum of apoB in the apoC-III-containing and apoC-III-deficient fractions after immuno-affinity column chromatography. We used immuno-affinity chromatography with anti-apoC-III antibody (Academy Biomedical Inc, Houston, TX) bound to Sepharose 3B resin (Sigma-Aldrich, St. Louis, MO) to separate the plasma into fractions with and without apoC-III. Concentrations of apoA-I and apoB in the apoC-III-containing and deficient fractions were assessed by sandwich ELISA using polyclonal antibodies (Academy Biomedical Inc, Houston, TX).

Upon precipitation of the apoB-containing lipoproteins from the apoC-III based immuno-fractions, we assessed the concentrations of apoC-III in HDL and cholesterol in HDL. Liquid transfer for 96-well plate loading and ELISA dilutions were handled robotically with a Multiprobe II (Perkin Elmer, Waltham, MA) to minimize pipetting error. Both ELISA and lipids plates were read with a BioTek ELx808iu 96-well plate reader controlled by KCJunior software (BioTek, Winooski, VT). All assays were completed in triplicate and any sample with an intra-assay coefficient of variation over 15% was repeated. Laboratory personnel were blinded to the case status. Our laboratory has within-run average CVs of 8% for HDL-C without apoC-III, 13% for HDL-C with apoC-III, 10% for apoAI with apoC-III, 7% for apoAI without apoC-III, and 14% for apoC-III in HDL. The detection ranges are (in mg/dL): apoA-I with apoC-III = 1-40; apoA-I without apoC-III = 10-350; apoC-III = 0.5-50.

Each 96-well plate included two control samples with known values that were subsequently used to standardize the plate. All samples analyzed together on one plate were considered a batch. Statistical analyses of the association between the HDL subtypes with risk of CHD additionally took batch variation into account by including batch in the strata statement. This was important because some batches were composed of 90% CHD cases.

In the DCH study, 161 samples of the original 3,830 sample case cohort set did not have enough samples left for lipid analyses. We also excluded 27 samples that did not pass quality control, so the final the case-cohort set included 1,949 incident CHD cases and 1,693 non-cases. Due to the case-cohort design, 57 incident CHD cases also belonged to the reference sub-cohort (total n=1,750).

Laboratory methods: MESA

Samples were removed from -80C storage and thawed at room temperature. Whole plasma was fractionated into lipoproteins containing apoC-III and those deficient of apoC-III by immuno-affinity separation on a 96-well microplate (Greiner Bio-One MICROLON™ 600, VWR Cat #82050-734) coated with rabbit anti-human apoC-III antibody (Academy Biomedical, Cat #33A-R1b, 10 mcg/mL in 1xPBS). Following overnight incubation at 4°C, the unbound fraction depleted of apoC-III-containing lipoproteins was collected for analysis. After washing with 1x PBS, ELISA diluent (1xPBS/2% BSA/0.05% Tween 20) was added to each well to dissociate the components of the bound apoC-III-containing lipoproteins from the plate during a 2hr incubation at 37°C and was collected for analysis. The concentrations of apolipoproteins were measured by sandwich ELISA using polyclonal antibodies (Academy Biomedical Company, Houston TX): apoC-III in whole plasma (coating antibody Cat# 33A-R1b at 10 mcg/mL in 1xPBS, detection antibody Cat# 33H-G2b at 1 mcg/mL in 1xPBS); and apoA-I in HDL with apoC-III and in HDL without apoC-III (coating antibody Cat #11A-G2b at 5 mcg/mL in 1xPBS, detection antibody Cat #11H-G1b at 1 mcg/mL in 1xPBS). For all ELISAs, the coating antibody was incubated for 1 hour at 37°C. Plates were washed three times with washing buffer (0.1% Tween 20 in 1xPBS) then blocked (Pierce, Casein in 1xPBS at 1% w/v, VWR Cat #PI37528) with 1 hour incubation at 37°C, followed by washing three times with washing buffer. Three extensively characterized plasma pools whose apoA-I concentrations in whole plasma and the apoC-III plasma fractions had been calibrated using commercially available standards and immuno-affinity column chromatography were used, one for the calibration curve and the other two as known-concentration controls to assess batch validity and between-batch variance. The calibration curve was prepared in dilutions starting at 10,000x and serially 2x further to 640,000x in 1xPBS containing 0.5% BSA creating a reliable second degree polynomial curve fit. The calibration

curve, known controls, and unknown samples were diluted in 1xPBS containing 0.5% BSA and incubated overnight at 4°C or 1 hr at 37°C. Following incubation, plates were washed three times with wash buffer, appropriate detection antibody was added as described, plates were incubated 1 hr at 37°C, and then washed three times with wash buffer. Avidin peroxidase was then added (Sigma Aldrich, Cat #A7419-2ML, 0.01 μg/ml in 1xPBS) and plates were incubated for 1 hr at 37°C then washed three times with wash buffer. Finally, o-phenylenediamine (OPD) (Sigma Aldrich, Cat #P9187-50SET) was added to all plates to develop color for 1 hour and 20 minutes at room temperature and the absorbance was read at 450 nm. Each sample was measured in duplicate. All laboratory personnel were blinded to the case status. Using this methodology, the within-run average CVs were 5% for apoA-I without apoC-III, 8% for apoA-I with apoC-III, and 4% for total apoC-III. The detection ranges are (in mg/dL): apoA-I with apoC-III = 1-40; apoA-I without apoC-III = 10-350; apoC-III = 0.5-50."

Upon excluding 45 participants with implausible apolipoprotein ranges; 27 with missing information on CHD events; 66 with missing covariate data, 5,658 MESA participants remained for our analyses.

To account for moderate variation in apolipoprotein levels by batch, values of each apolipoprotein exposure were recalibrated to represent the average distribution across batches (Rosner et al. 2008). Log-transformed apolipoprotein variables were regressed on batch and age as well as other variables associated with apolipoprotein levels (sex, race, study site, smoking status, alcohol intake, education, and BMI) using linear regression models. We used the back-transformed measures of HDL-apoA-I with and without apoC-III in our analyses.

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Supplemental tables and figures

Table S1. Partially adjusted Spearman correlations between HDL subspecies (apoA-I based) and other lipid measures (MESA cohort).

Table S2. Results from analyses with HDL-cholesterol (rather than apoA-I concentrations) containing and lacking apoC-III in the DCH.

Table S3. Published results for HDL-cholesterol containing and lacking apoC-III in the Nurses' and Health Professionals Follow-Up Study (published in JAHA 2012) – adjusted results shown here used for meta-analysis of all 4 cohorts.

Table S4. Coronary heart disease risk per 5 mg/dL increment higher total HDL, HDL lacking apoC-III and containing apoC-III in the 4 studies.

Table S5. Medians of the study-specific quintiles meta-analyzed in figure 2.

Table S6. Results across race/ethnicity in the MESA study.

Fig S1 and S2. Forest plots: Fixed-effects meta-analysis of relative risks per 1 SD with 95% CI for risk of CHD estimated in four prospective studies for HDL containing and lacking apoC-III.

Fig S3. Coronary heart disease risk associated with the *proportion* of HDL that contains apoC-III and with total HDL in four prospective studies (meta-analysis).

Table S1. Spearman correlations between ApoA-I subspecies and other lipid measures (MESA cohort).

	ApoAI containing apoC-III	**Total apoC-III**	**Proportion of apoAI that contains apoC-III**	**ln_triglycerids**
ApoAI lacking apoC-III	0.67 <.0001	0.30 <.0001	-0.03 0.0255	-0.16 <.0001
ApoAI containing apoC-III	1.00000	0.42 <.0001	0.67 <.0001	-0.019 0.1558
Total apoC-III		1.00000	0.28 <.0001	0.62 <.0001
Proportion of apoAI that contains apoC-III			1.00000	0.12 <.0001

Partially adjusted spearman correlations: Age and sex included as covariates.

Table S2. Hazard Ratios (HRs) and 95% confidence intervals of CHD according to quintiles of HDL-cholesterol without and with apoC-III in the Diet, Cancer and Health Cohort

	Q1	**Q2**	**Q3**	**Q4**	**Q5**	**P trend**
HDL-cholesterol lacking apoC-III						
Median levels in sub-cohort (mg/dL)	*25.3*	33.8	41.7	51.2	68.1	
Number of cases	*566*	378	378	308	319	
Crude model	*1.0 (ref)*	0.57(0.43-0.75)	0.55 (0.42-0.73)	0.42 (0.31-0.56)	0.33 (0.24-0.44)	<0.0001
Multivariable model	*1.0 (ref)*	0.64 (0.49-0.85)	0.59 (0.44-0.79)	0.54 (0.39-0.73)	0.41 (0.30-0.58)	<0.0001
HDL-cholesterol containing apoC-III						
Median levels in sub-cohort (mg/dL)	*2.9*	5.3	7.6	10.3	15.8	
Number of cases	*433*	337	400	354	425	
Crude model	*1.0 (ref)*	0.79 (0.57-1.11)	0.91 (0.63-1.30)	0.75 (0.51-1.11)	0.81 (0.53-1.24)	0.3
Multivariable model	*1.0 (ref)*	0.84 (0.59-1.19)	1.03 (0.71-1.50)	0.89 (0.59-1.32)	0.90 (0.58-1.40)	0.7

Hazard Ratios (HRs) obtained from Cox proportional hazard regression models. P for trend using the medians of the quintiles. Multivariable model: sex, smoking, education, alcohol, BMI, postmenopausal status, (women), and hypertension. HDL-cholesterol with and without apoC-III are simultaneously included in all models.

Table S3. Incidence rate ratios (IRR) and 95% confidence intervals of CHD according to quintiles of total HDL-cholesterol, HDL-cholesterol containing and lacking apoC-III in the Nurses' Health Study (NHS) and the Health Professionals Follow-Up Study (HPFS).

	Q1	Q2	Q3	Q4	Q5	P trend
			Total HDL-cholesterol			
NHS (median; mg/dL)	44.0	59.1	69.9	81.9	103.5	
Multivariate	*1.0 (ref)*	0.88 (0.50-1.54)	0.68 (0.36-1.30)	0.79 (0.41-1.51)	0.41 (0.18-0.92)	0.05
HPFS (median, mg/dL)	31.3	40.2	47.5	54.8	69.1	
Multivariate	*1.0 (ref)*	0.59 (0.36-0.97)	0.68 (0.40-1.16)	0.54 (0.30-0.96)	0.53 (0.28-1.01)	0.07
			HDL-cholesterol lacking apoC-III			
NHS (median; mg/dL)	36.7	51.0	61.4	72.59	92.3	
Multivariate	*1.0 (ref)*	1.03 (0.54-1.95)	0.68 (0.33-1.42)	0.74 (0.33-1.65)	0.30 (0.12-0.77)	0.006
HPFS (median, mg/dL)	27.4	38.6	42.1	49.0	62.2	
Multivariate	*1.0 (ref)*	0.38 (0.21-0.67)	0.61 (0.34-1.09)	0.35 (0.19-0.66)	0.32 (0.16-0.66)	0.03
			HDL-cholesterol containing apoC-III			
NHS (median; mg/dL)	4.6	6.2	8.88	10.8	15.1	
Multivariate	*1.0 (ref)*	0.78 (0.41-1.46)	1.12 (0.60-2.11)	1.00 (0.51-1.99)	1.44 (0.69-3.01)	0.14
HPFS (median, mg/dL)	2.7	3.86	5.0	6.2	8.5	
Multivariate	*1.0 (ref)*	1.10 (0.64-1.89)	1.32 (0.74-2.33)	1.19 (0.65-2.19)	1.76 (0.94-3.31)	0.08

Incidence rate ratios (IRR) obtained from conditional logistic regression models (matched on age and smoking) adjusted for alcohol, BMI, self-reported diagnosis of hypertension before blood draw, and postmenopausal status and hormones in NHS only. HDL-cholesterol with and without apoC-III are simultaneously included in all models. P trend is the test for linear trend across quintiles.

Table S4. Coronary heart disease risk per 5 mg/dL increment higher HDL lacking apoC-III and containing apoC-III in the four studies.

	MESA	DCH	NHS	HPFS	Pooled*
HDL lacking apoC-III	0.97 (0.95, 0.99)	0.97 (0.95, 0.98)	0.88 (0.81, 0.95)	0.92 (0.85, 0.99)	0.97 (0.96, 0.98)
HDL containing apoC-III	1.14 (0.92, 1.41)	1.02 (0.95, 1.10)	1.46 (1.07, 1.99)	1.33 (0.90, 1.98)	1.06 (0.99, 1.13)

Hazard Ratios and 95% confidence intervals for coronary heart disease per 5 mg/dL increment.

Multivariable model adjusted for ASCVD risk factors: age, sex, prevalent diabetes, treatment with anti-hypertensive medication, systolic blood pressure, smoking, and total cholesterol. Both subtypes of HDL (containing and lacking apoC-III) are simultaneously included.

*Study-specific estimates combined using fixed-effects meta-analysis.

Table S5. Medians of the study-specific quintiles meta-analyzed in figure 1:

	Q1	**Q2**	**Q3**	**Q4**	**Q5**
ApoA-I lacking apoC-III (mg/dL)					
Median, MESA	86.3	104.3	117.5	133.2	164.8
Median, DCH sub-cohort	82.0	105.7	123.5	145.9	181.5
***ApoA-I containing apoC-III* (mg/dL)**					
Median, MESA	5.0	6.6	7.9	9.4	12.3
Median, DCH sub-cohort	5.0	8.4	11.3	14.8	21.2
HDL cholesterol lacking apoC-III (mg/dL)					
Median, NHS	36.7	51.0	61.4	72.59	92.3
Median, HPFS	27.4	38.6	42.1	49.0	62.2
***HDL cholesterol containing apoC-III* (mg/dL)**					
Median, NHS	4.6	6.2	8.88	10.8	15.1
Median, HPFS	2.7	3.86	5.0	6.2	8.5

Table S6. Coronary heart disease risk per standard deviation of apoA-I with and without apoC-III in strata of race/ethnicity in the Multi-Ethnic Study of Atherosclerosis.

Self-reported race/ethnicity	HR for apoA-I lacking apoC-III*	HR for apoA-I containing apoC-III*	P heterogeneity
Caucasian (n=2102)	0.67 (0.53, 0.85)	1.09 (0.88, 1.36)	0.02
Chinese American (n=694)	1.00 (0.51, 1.96)	0.81 (0.41, 1.56)	0.7
African American (n=1617)	0.88 (0.65, 1.18)	1.05 (0.79, 1.39)	0.5
Hispanic (n=1244)	0.80 (0.56, 1.13)	0.93 (0.65, 1.34)	0.6

*Hazard Ratios and 95% confidence intervals for coronary heart disease per 1 SD increments.

Multivariable model adjusted for ASCVD risk factors: age, sex, prevalent diabetes, treatment with anti-hypertensive medication, systolic blood pressure, smoking, and total cholesterol. ApoA-I with and without apoC-III are simultaneously included in all models

Fig S1 and S2. Forest plots for fixed-effects meta-analysis of relative risks with 95% CI for risk of CHD estimated per study-specific SDs in four prospective studies (the Multi-Ethnic Study of Atherosclerosis [MESA], the Diet, Cancer and Health [DCH] Study, the Nurses' Health Study [NHS], and the Health Professionals Follow-Up Study [HPFS]) per SD and using study-specific quintiles. Adjusted for ASCVD risk factors: age, sex, prevalent diabetes, treatment with anti-hypertensive medication, (race/ethnicity in MESA), systolic blood pressure, smoking, and total cholesterol. Both subtypes of HDL (containing and lacking apoC-III) are simultaneously included in all models.

Fig S1 Coronary heart disease risk associated per SD of HDL containing apoC-III (study specific meta-analysis RR per SD of four prospective studies).

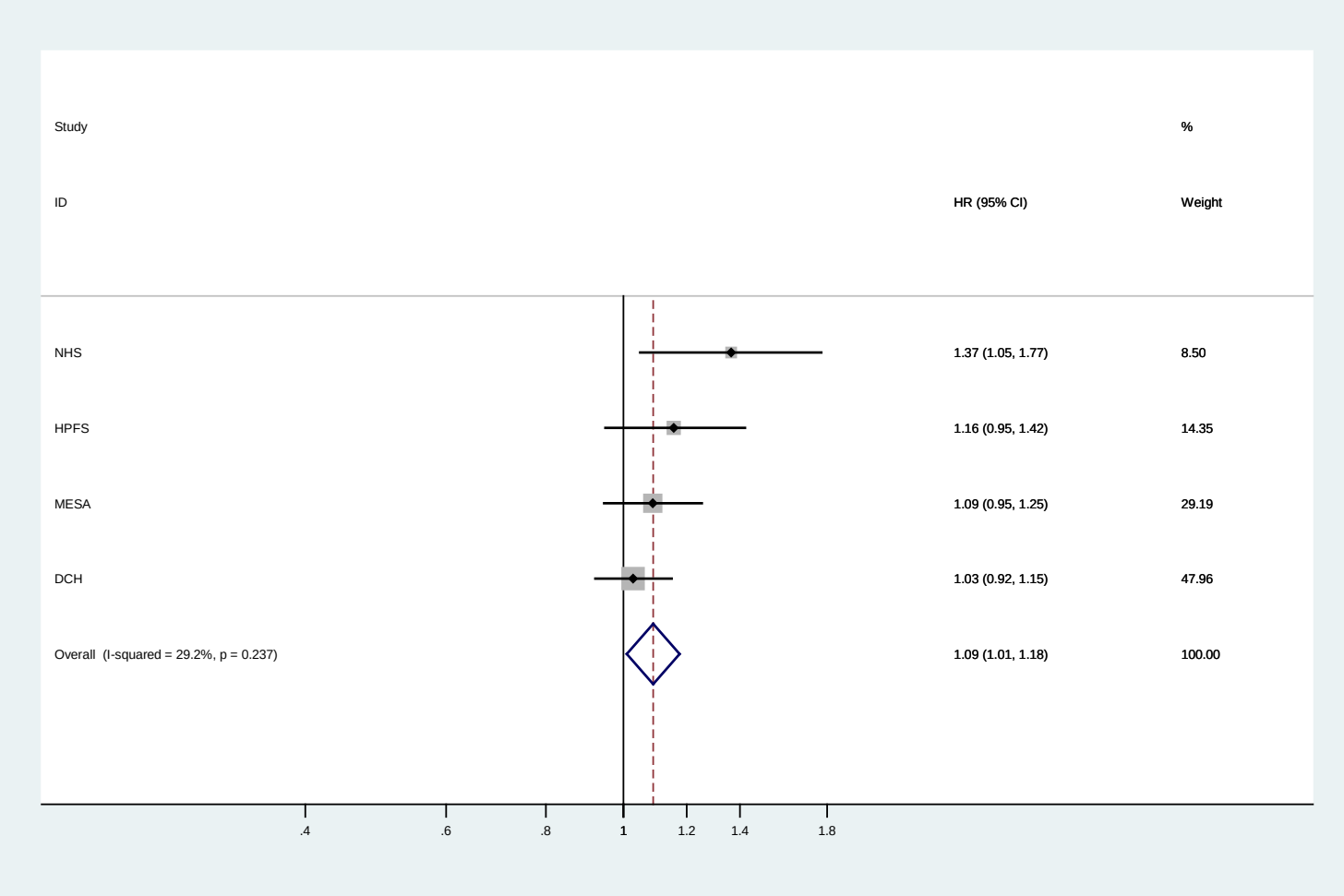


Fig S2 Coronary heart disease risk associated per SD of HDL lacking apoC-III (study specific meta-analysis RR per SD of four prospective studies).

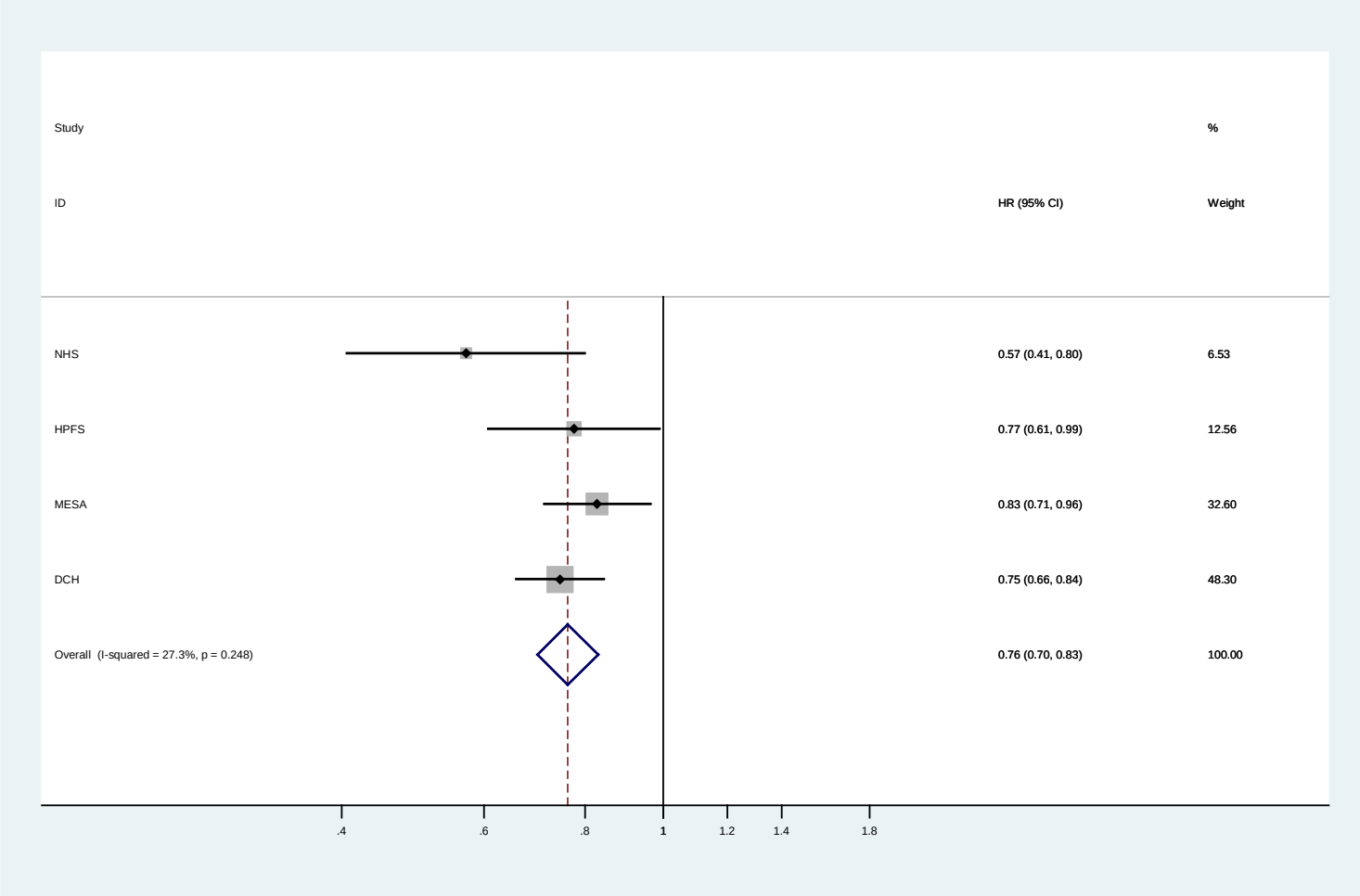
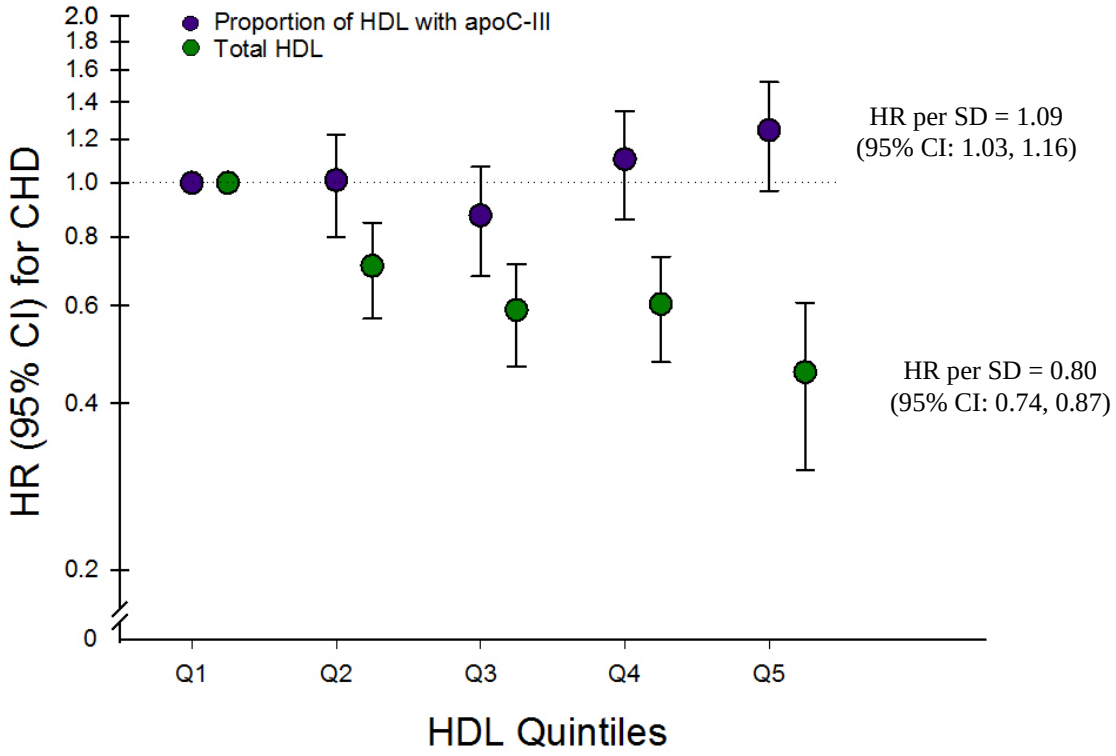


Fig S3. Coronary heart disease risk associated with proportion of HDL that contains apoC-III and with total HDL in four prospective studies



Pooled relative risks with 95% CI for risk of CHD estimated in fixed-effects meta-analysis using study-specific SDs in four prospective studies (the Multi-Ethnic Study of Atherosclerosis [MESA], the Diet, Cancer and Health [DCH] Study, the Nurses' Health Study [NHS], and the Health Professionals Follow-Up Study [HPFS]). Adjusted for ASCVD risk factors: age, sex, prevalent diabetes, treatment with anti-hypertensive medication, (race/ethnicity in MESA), systolic blood pressure, smoking, and total cholesterol. HDL=per apoA-I concentrations in MESA and DCH; per HDL-cholesterol concentration in NHS and HPFS.