Supplemental Material

Paradoxical association of enhanced cholesterol efflux with increased incident cardiovascular risks

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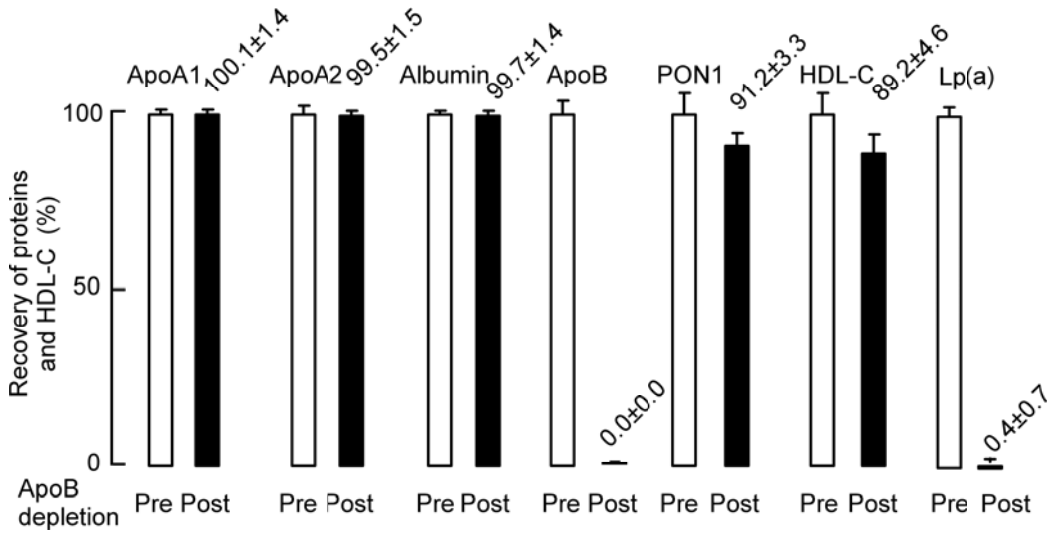
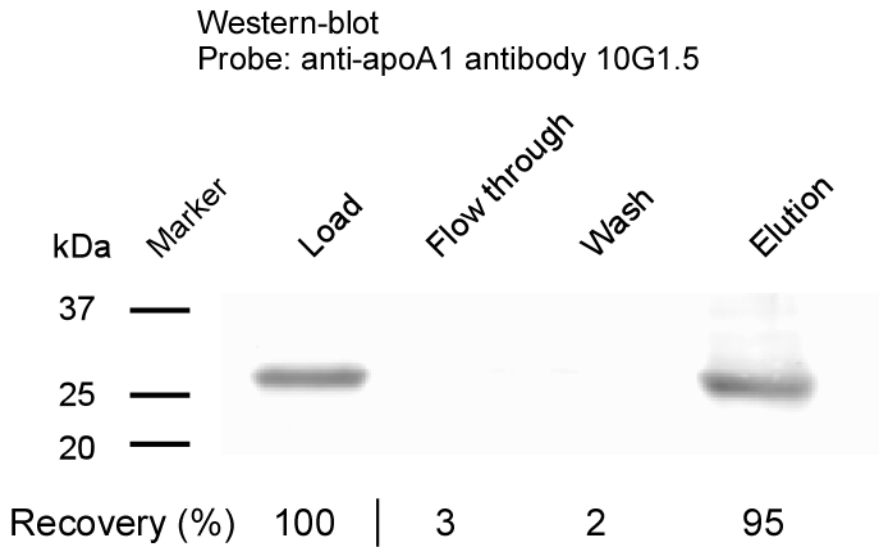
Supplemental Figure I
Recovery of ApoA1, ApoA2, albumin, ApoB, PON1, HDL cholesterol and Lp(a) after serum ApoB-depletion.



Serum samples from the outpatient cohort were treated with PEG 6000 to form ApoB-depleted serum as described under Methods. For ApoA1, ApoA2, albumin, ApoB, PON 1 and HDL cholesterol, data shown are from 20 random individual sera before and after ApoB-depletion. For Lp(a), 12 individual sera from the angiographic cohort with elevated Lp(a) levels ranging from 35 to 133 mg/dL were used, and Lp(a) levels were measured before and after PEG treatment. Results shown represent mean +/- SD.

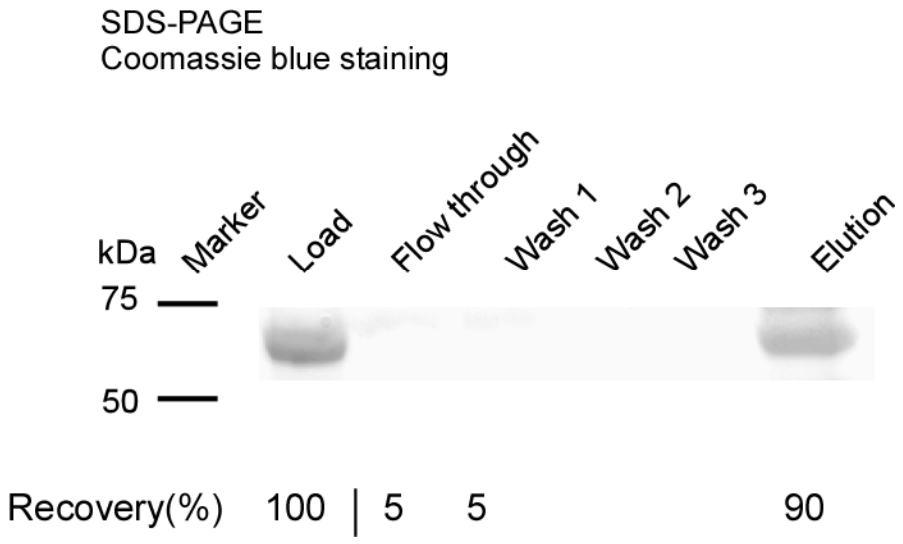
Supplemental Figure II

Western-blot of load, flow through, wash and elution of immunoprecititation of ApoA1 from lipoprotein-deficient medium and quantitative ApoA1 recovery.



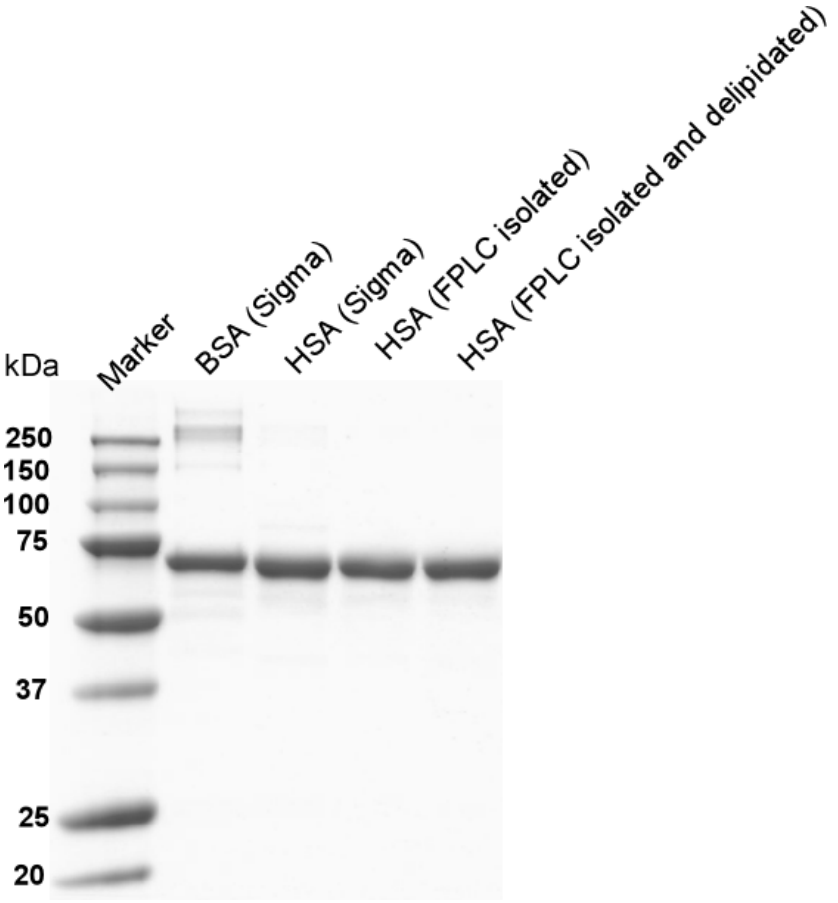
In this study we sought to confirm quantitative recovery of ApoA1 under the conditions employed to determine the amount of radiolabeled-cholesterol associated with ApoA1. Briefly, media used in cholesterol efflux activity studies was first separated by buoyant density centrifugation into various lipoprotein fractions vs. the lipoprotein depleted media (LPDM, d>1.21). The LPDM was then mixed with a cocktail of monoclonal antibodies specific to ApoA1 as described under Methods. Following brief incubation, this mixture was then applied to an immobilized Protein A resin column. The flow through fraction, wash and eluted fraction were collected, volumes measured, and both radiolabeled cholesterol, and the abundance of ApoA1 within each fraction determined. Shown above are the Western blot analyses of the indicated fractions analyzed by SDS PAGE using antibody specific for ApoA1. The calculated % recovery of ApoA1 from the starting material (100% loading) is indicated on the Figure. Note that 97% of the ApoA1 was removed from the starting (load) material, and 95% of the ApoA1 was recovered in the eluted fraction under the conditions employed to determine the proportion of cholesterol bound to ApoA1 during the cholesterol efflux studies.

Supplemental Figure III
SDS-PAGE of load, flow through, wash and elution of immunoprecipitation of albumin from lipoprotein-deficient medium and quantitative albumin recovery.



In this study we sought to confirm quantitative recovery of albumin under the conditions employed to determine the amount of radiolabeled-cholesterol associated with albumin. Briefly, lipoprotein depleted media from cholesterol efflux activity studies was mixed with a cocktail of polyclonal antibodies specific to human albumin as described under Methods. This mixture was then applied to an immobilized Protein A/G resin column. The flow through fraction, wash and eluted fraction were collected, volumes measured, and both radiolabeled cholesterol, and albumin abundance within each fraction determined. Shown above are the analyses of the indicated fractions analyzed by SDS PAGE. The calculated % recovery of albumin from the starting material (100% loading) is indicated on the Figure. Note that 95% of the albumin was removed from the starting (load) material, and 90% of the albumin was recovered in the eluted fraction under the conditions employed to determine the proportion of cholesterol bound to albumin (or albumin associated protein(s)) during the cholesterol efflux studies.

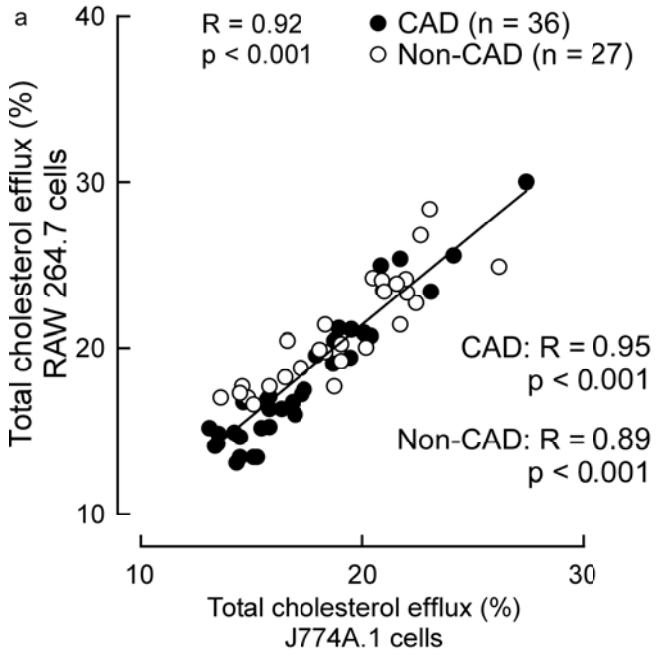
Supplemental Figure IV
SDS-PAGE of various sources of albumin (listed left to right from the Marker lane) including Bovine Serum Albumin (BSA) (Sigma), Human Serum Albumin (HSA) (Sigma), FPLC isolated HSA and FPLC isolated and delipidated HSA. Each sample lane was loaded with 5 μg protein.

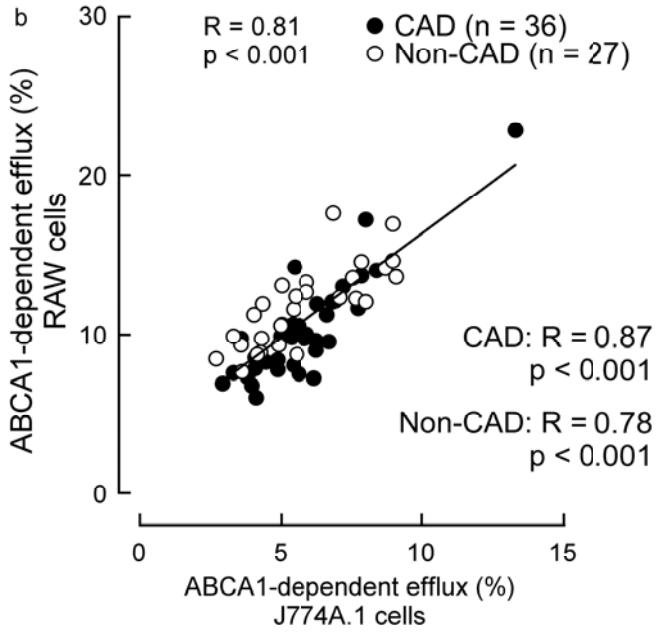


The purity of HSA and BSA were assessed from the indicated sources by analyzing comparable amounts of protein per lane fractionated by SDS PAGE and detected with Coomassie Blue staining. Human albumin (FPLC isolated +/- delipidation) was isolated as described under Methods**.**

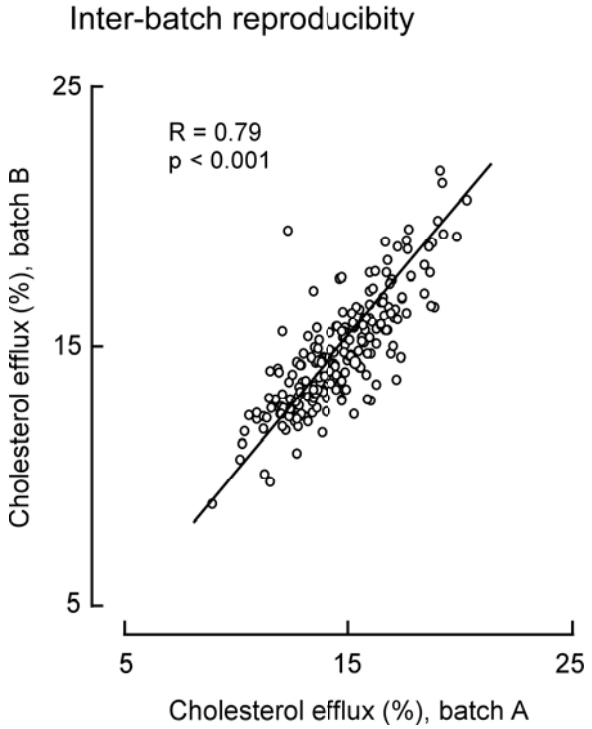
Supplemental Figure V

Comparison of ApoB-depleted serum cholesterol efflux activity from RAW 264.7 cells and J774A.1 cells. Cellular cholesterol efflux assays were performed under conditions of ABCA1 stimulation as described in Methods using murine macrophage RAW 264.7 cells and J774A.1 cells for direct head-to-head comparisons. For these studies, serum was used from a random sampling of subjects with documented CAD (n=36) and subjects without known CAD (n=27) to determine whether comparable efflux activity results were observed among both disease and non-disease subjects. All data points represent duplicate experiments. R = Spearman correlation coefficient.



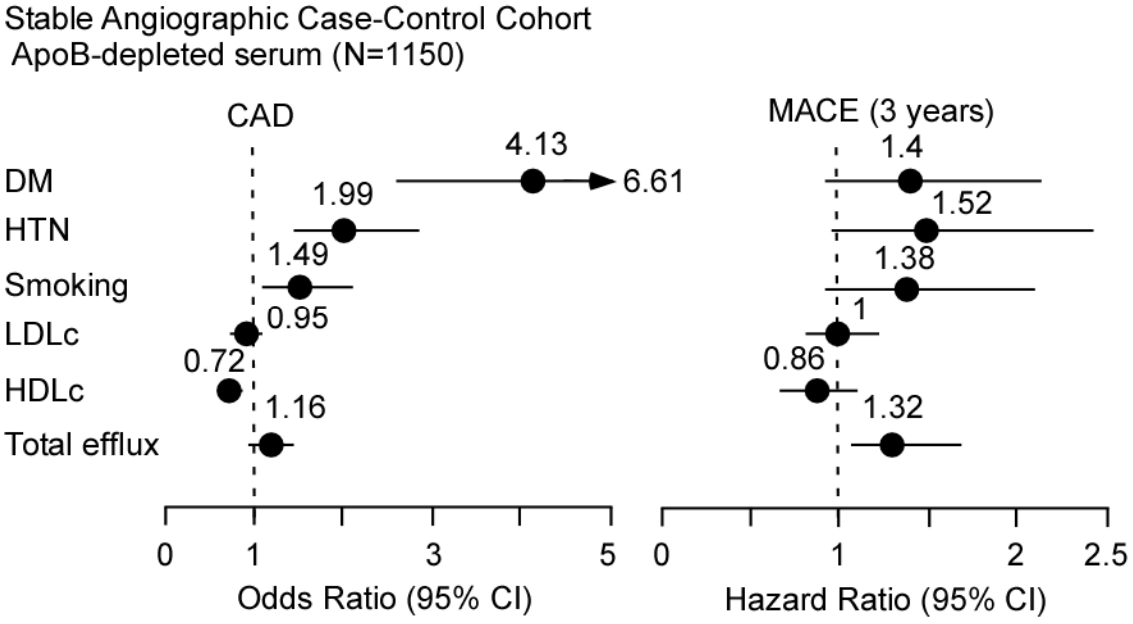


Supplemental Figure VI
Inter-batch ApoB-depleted serum total cholesterol efflux assay reproducibility. Pools of serum representing low, medium and high HDL-c levels were used in ABCA1 stimulated cholesterol efflux assays as described in Methods. Data points represent at least triplicate determinations. Results were plotted as month-month variability. R = Spearman correlation coefficient.



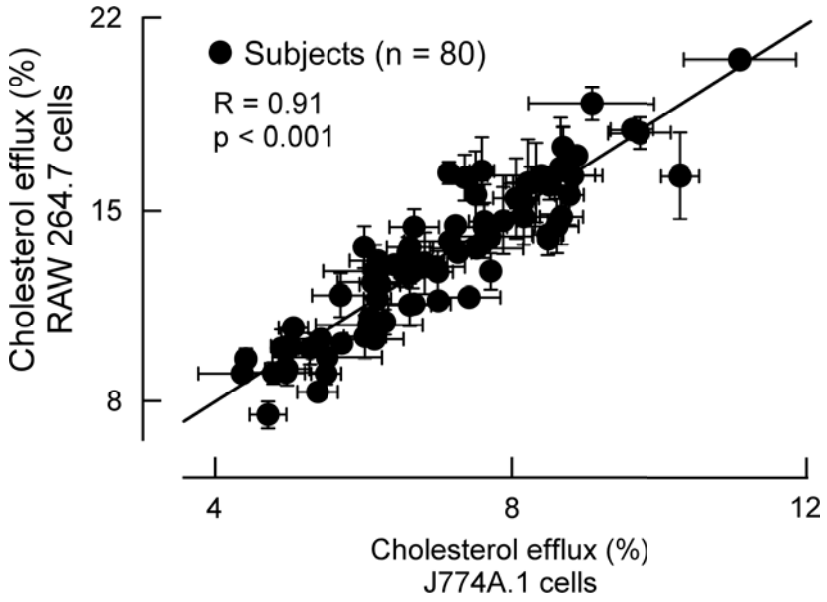
Supplemental Figure VII

Odds ratios for prevalent coronary artery disease risk, and hazard ratios for incident major adverse cardiovascular risk according to cholesterol efflux activity. ApoB depleted serum from subjects in the Stable Angiographic Cohort were assayed for cholesterol efflux activity using ApoB-depleted serum as cholesterol acceptor. Coronary artery disease (CAD); Results (n=1150) were fit to a multiple logistic regression model with DM, HTN, smoking, LDLc, HDLc, age, gender, total efflux. Major adverse cardiac events within 3 years (MACE3); Results were fit to a Cox model with DM, HTN, smoking, LDL, HDL, age, gender, total efflux. The 5-95% confidence interval is indicated by line length.



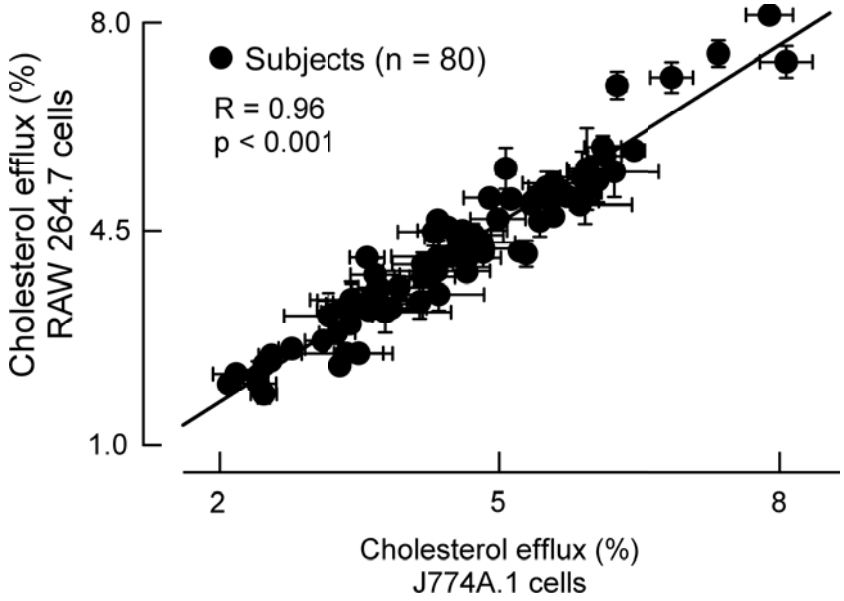
Supplemental Figure VIII

Direct comparison of total cholesterol efflux activity between RAW264.7 and J774A.1 cell lines. Cellular cholesterol efflux activity was measured under conditions of ABCA1 stimulation as described in Methods using either murine macrophage RAW 264.7 cells or J774A.1 cells for direct comparisions. For these studies, ApoB-depleted serum was used from a set of 80 random subjects. Each point represents the mean and range of replicate determinations for each cell type.



Supplemental Figure VIIII

Direct comparison of cAMP-independent cholesterol efflux activity between RAW264.7 and J774A.1 cell lines. Cellular cholesterol efflux activity was measured in the presence vs absence of ABCA1 stimulation as described in Methods using either murine macrophage RAW 264.7 cells or J774A.1 cells for direct comparisions. ABCA1-independent cholesterol efflux activity is shown. For these studies, ApoB-depleted serum was used from a set of 80 random subjects. Each point represents the mean and range of replicate determinations for each cell type.



Supplemental Table I
HDL related parameters for sera used to quantify radiolabel cholesterol distribution in efflux assay

	HDLc (mg/dL)	ApoA1 (mg/dL)	LDLc (mg/dL)	Total cholesterol (mg/dL)
Pooled sera A	67.4	173.0	119.0	200.2
Pooled sera B	59.3	156.1	104.0	180.9
Pooled sera C	56.8	151.8	111.0	186.5
Pooled sera D	61.4	163.3	111.0	193.0

These 4 sera pools represent data from pooled sera from multiple apparently healthy volunteer subjects, each pool comprised of ~5 units of serum. They were used to monitor the lipoprotein compartments into which radiolabeled cholesterol partitions during the cellular cholesterol efflux activity assay and were also used as controls during efflux activity assays. These were aliquoted and were used after single thaw in every efflux assay performed in this report to ensure consistency. Their HDLc, ApoA1, LDLc and total cholesterol levels were similar to that of the healthy control subjects described in the outpatient cohort. HDLc, ApoA1, LDLc and total cholesterol concentrations were determined as described in Methods.

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Supplemental Table II

Comparison of percentage distribution of ApoA1 in the fraction of HDL (1.063<d<1.21) and the fraction having d>1.21 with versus without PEG.

	HDL (control)	HDL+PEG
ApoA1 in HDL (%) (1.063<d<1.21)	87.7 ± 0.2	89.2 ± 0.2
ApoA1 in d>1.21 (%)	12.3 ± 0.2	10.8 ± 0.2

Samples were processed and ApoA1 determined as described in Methods.

Supplemental Table III
Predicated cholesterol efflux acceptor activity and contribution in serum by ApoA1 and albumin based upon specific activity of cholesterol efflux activity and normal circulating concentrations

	Specific activity of cholesterol efflux [%/(mg/ml) /15 hrs]	serum concentration (mg/ml)	Predicted total cholesterol efflux activity in serum (% /15 hrs)	% contribution to total predicted cholesterol efflux activity in serum
ApoA1	2075.0	1.5	3112.5	90.4
Albumin	7.3	45.0	329.0	9.6
Fold	ApoA1 is 284 fold better cholesterol acceptor than albumin	Albumin is 30-fold more abundant than ApoA1		At normal serum levels, albumin is predicted to account for ~1/10th as much cholesterol acceptor activity as ApoA1

Supplemental Table IV
Correlation Coefficients between HDL/Lipoproteins-Related Variables and ApoB-depleted Serum Total Cholesterol Efflux

Variable	Outpatient and Healthy-Volunteer Cohort		Angiographic cohort			
	Non-CAD (N=431)	CAD (N=146)	Non-CAD (N=279)	CAD (N=871)	MI/Stroke (N=58)	MACE (N=113)
HDLc	0.55 (p<0.001)	0.31 (p<0.001)	0.55 (p<0.001)	0.41 (p<0.001)	0.3 (p=0.023)	0.42 (p<0.001)
ApoA1	0.63 (p<0.001)	0.43 (p<0.001)	0.58 (p<0.001)	0.55 (p<0.001)	0.53 (p<0.001)	0.62 (p<0.001)
ApoA2	0.47 (p<0.001)	0.55 (p<0.001)	0.49 (p<0.001)	0.45 (p<0.001)	0.46 (p<0.001)	0.53 (p<0.001)
Albumin	-0.05 (p=0.322)	0.19 (p=0.064)	0.05 (p=0.394)	0.07 (p=0.06)	-0.08 (p=0.54)	0.07 (p=0.457)