

## Supplementary Appendix

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## Supplementary Appendix

# HDL Cholesterol Efflux Capacity and Incident Cardiovascular Events

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## **Supplementary Methods**

### **Study Population**

Enrollment in the Dallas Heart Study (DHS) occurred between July 2000 and September 2002, and data collection was performed in 3 phases, beginning with an initial in-home visit (n = 6101) to collect demographic information, medical history, blood pressure, and anthropometric measurements.<sup>1</sup> Participants aged 30 to 65 years were asked to undergo a second in-home visit (N=3399) to obtain fasting blood and urine collection and then return to UT Southwestern for a final visit (N=2,971) to obtain dual-energy x-ray absorptiometry (DEXA) scanning for body composition, detailed cardiovascular phenotyping by electron-beam computed tomography (EBCT) and cardiac magnetic resonance imaging (MRI), and abdominal MRI for body fat distribution.

### **Risk Factor Measurements**

Demographic information, anthropometric measurements, and other variables were collected at baseline and definitions have been described in detail previously.<sup>1</sup> Hypertension was defined as an average systolic blood pressure  $\geq 140$  mm Hg or diastolic blood pressure  $\geq 90$  mm Hg or use of antihypertensive medication. Diabetes was defined by a fasting glucose level  $\geq 126$  mg/dL, nonfasting glucose of  $>200$  mg/dL, or self-reported diabetes coupled with the use of any glucose lowering medication. Body mass index (BMI) was calculated based on measured height and weight; waist and hip circumference were measured directly at the time of cardiac imaging. Insulin sensitivity was estimated using the homeostasis model assessment insulin resistance index (HOMA-IR) calculated using the HOMA Calculator version 2.2.<sup>2</sup>

### **Circulating Biomarker Measurements**

All circulating biomarker measurements were obtained at baseline from plasma stored at  $-70^{\circ}\text{C}$  and were made from the same fasting plasma samples used for lipoprotein and cholesterol efflux capacity measurements. Assay measurement details have been described previously for the following markers: lipoprotein (a), adiponectin,<sup>3</sup> leptin,<sup>4</sup> high sensitivity C-reactive protein (hs-CRP),<sup>5</sup> interleukin-18,<sup>6</sup> and cystatin C.<sup>7</sup>

### **Imaging Measurements**

Electron beam computed tomography was performed in duplicate on a single scanner to assess coronary artery calcium (CAC), with results averaged.<sup>8</sup> Scans were scored by the Agatston method. DEXA was used to measure total body fat, lean mass, percentage body fat, truncal and lower body fat.<sup>9</sup> Visceral fat (intraperitoneal + retroperitoneal fat) was measured by a 1.5-T MRI using a prospectively designed and validated method of fat mass prediction from a single MRI slice at the L2-L3 intervertebral level.<sup>10</sup>

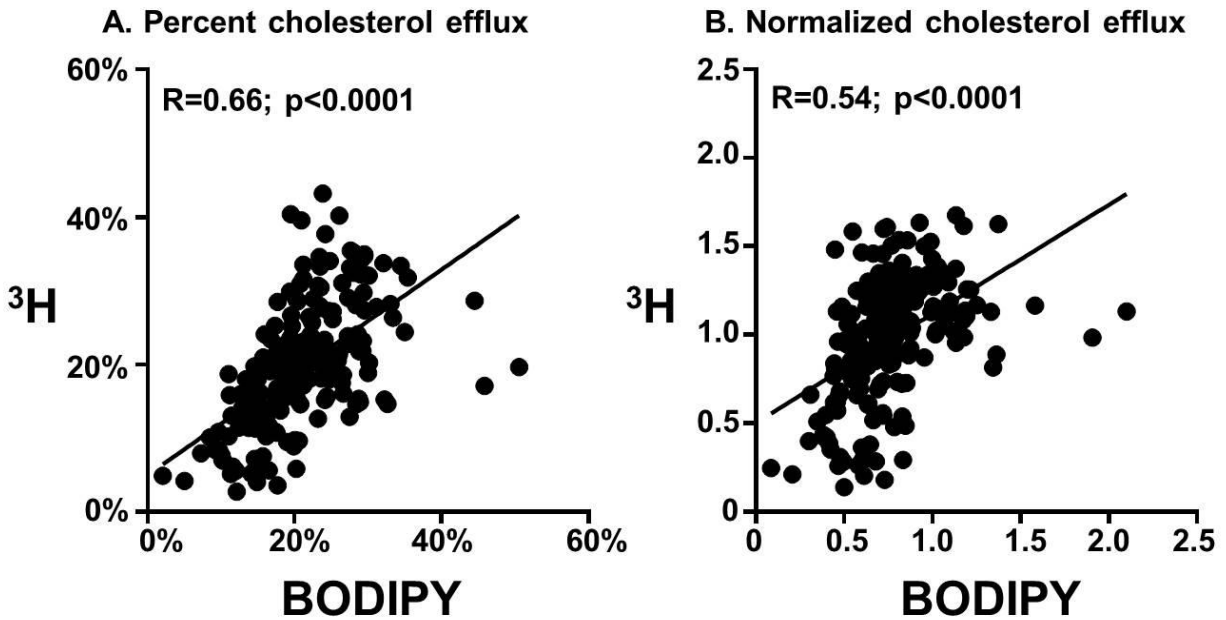
### **Cholesterol efflux capacity (CEC) measurements**

Macrophage-specific CEC was measured using BODIPY-cholesterol.<sup>11</sup> Briefly, J774 macrophages were dispensed into a 96-well plate at  $7 \times 10^4$  cells per well. The following day the cells were incubated for 1h with 0.025mM BODIPY-tagged cholesterol (Avanti Polar Lipids), 0.2% BSA, and 2 $\mu$ g/mL ACAT Inhibitor (Sandoz, Sigma-Aldrich) in RPMI plus 1% FBS. Following washing with MEM-HEPES, cells were incubated overnight in serum-free RPMI containing 0.3mM cAMP, 0.2% BSA, and 2 $\mu$ g/mL ACAT Inhibitor. Apolipoprotein B-depleted study subject plasma was prepared using PEG precipitation. After washing with MEM-HEPES, the BODIPY-cholesterol labeled cells were incubated with 2.8% apolipoprotein B-depleted plasma in MEM-HEPES buffer, 0.15mM cAMP and 2 $\mu$ g/mL ACAT Inhibitor for 4 hours at 37°C. The resulting quantity of BODIPY cholesterol in the media was determined with a spectrophotometer, and CEC was calculated as the amount of effluxed BODIPY cholesterol expressed as a fraction of the initial cell content of BODIPY cholesterol. Results were normalized to the measured efflux by a pooled reference apolipoprotein B-depleted plasma sample evaluated on every plate. All samples were run in duplicate and the average value was reported. Intra-plate CV was 3.3% and inter-plate CV was 7.4%. In a subset of 179 participants, CEC was also determined using radiolabeled cholesterol.<sup>12</sup>

## Supplementary Figure S1

### Correlation Between [<sup>3</sup>H]-cholesterol and BODIPY-Cholesterol Efflux Capacity.

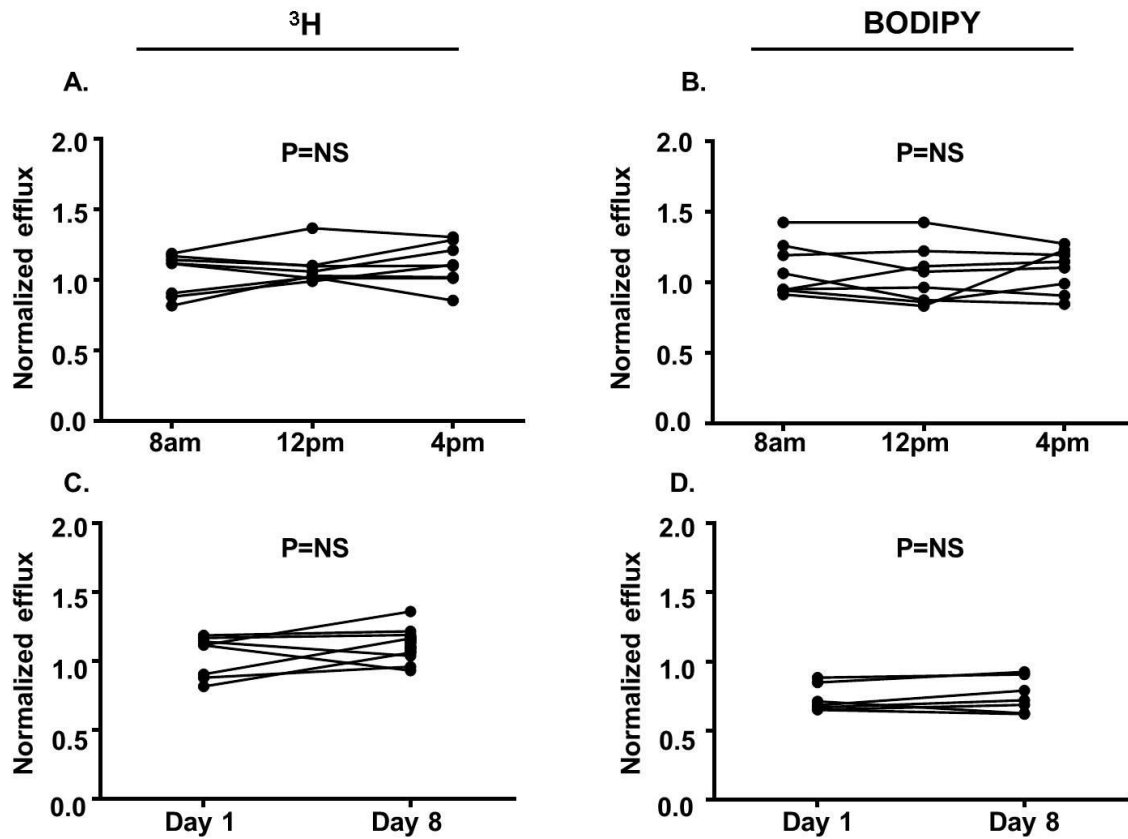
Participants from the Dallas Heart Study were randomly selected for efflux comparison studies (N=179). Fasting plasma samples were stored at -70°C. Cholesterol efflux capacity measured using radiolabeled cholesterol (<sup>3</sup>H) and fluorescent-labeled cholesterol (BODIPY) were compared expressed as percent efflux (A) or normalized to a reference sample (B) using Spearman correlation coefficients.



## Supplementary Figure S2.

### Temporal Variation in Cholesterol Efflux Capacity.

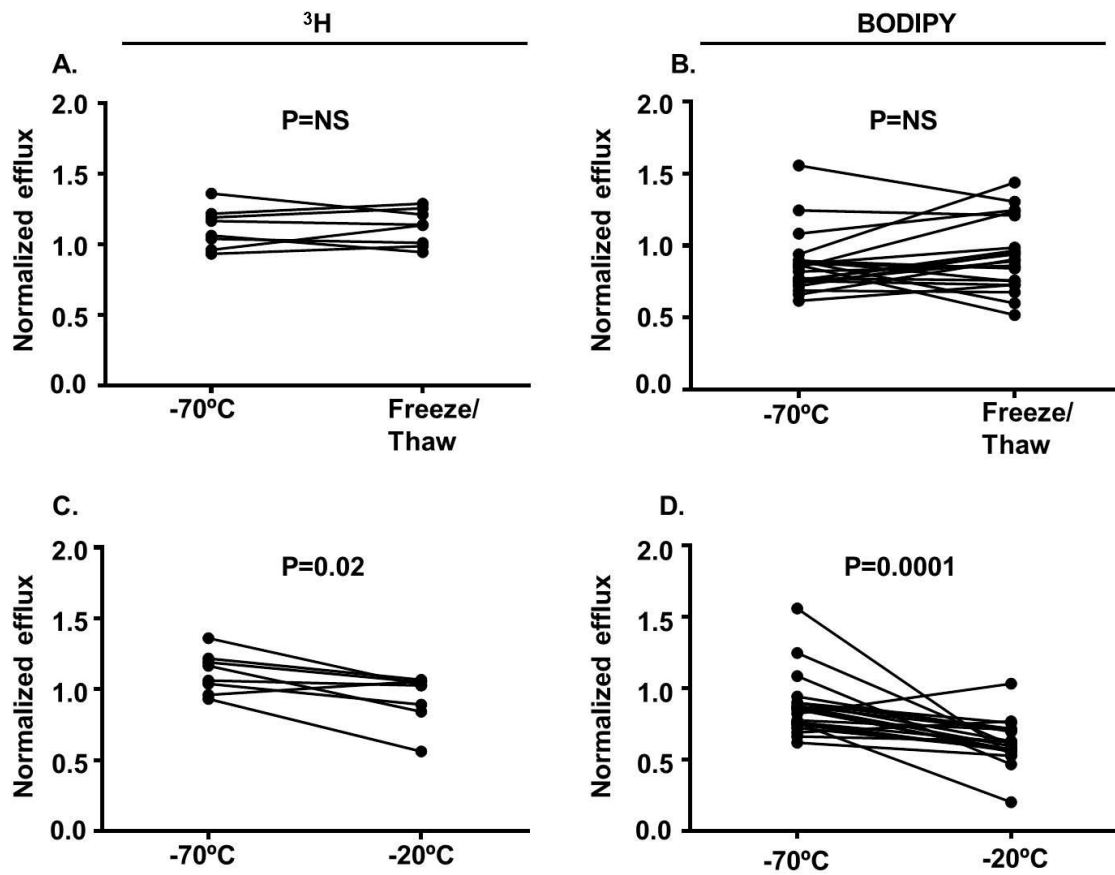
Plasma was collected from healthy men and women age >18 years (n=22) at 8am (fasting), 12pm, and 4pm on the same day (A, B), and 8am (fasting) 7 days later (C, D). Cholesterol efflux capacity measured using radiolabeled cholesterol ( $^3\text{H}$ ) (A, C) and fluorescent-labeled cholesterol (BODIPY) (B, D) was normalized to a reference sample.



### Supplementary Figure S3.

#### Effect of Storage Conditions on Cholesterol Efflux Capacity.

Plasma was collected from healthy men and women age >18 years (n=22) at 8am (fasting) and stored at -70°C, without or with one freeze/thaw cycle (A, B), or it was stored at -20°C for the same duration (3-12 months) (C, D). Cholesterol efflux capacity was measured using both radiolabeled cholesterol (<sup>3</sup>H) (A, C) and fluorescent-labeled cholesterol (BODIPY) (B, D).

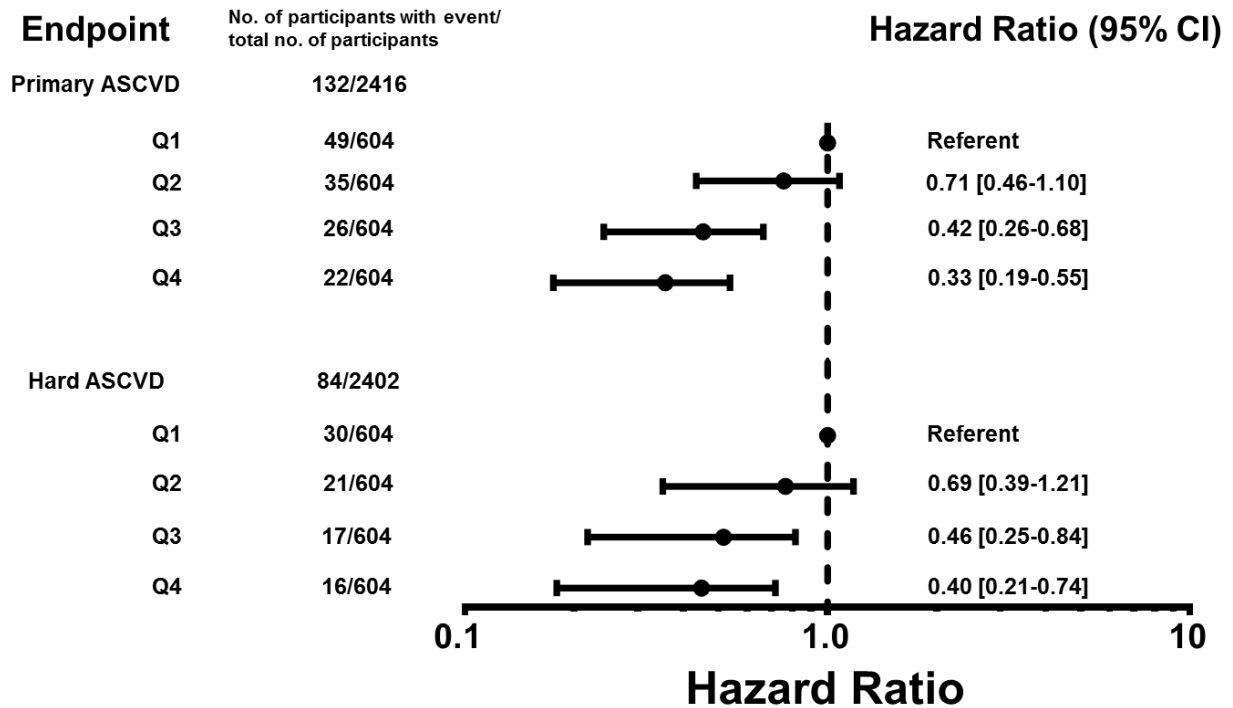




**Supplementary Figure S4.**

**Hazard Ratios for Quartiles of Cholesterol Efflux Capacity and the Primary Atherosclerotic Cardiovascular Disease Endpoint versus a Restricted Endpoint of Hard Atherosclerotic Cardiovascular Disease.**

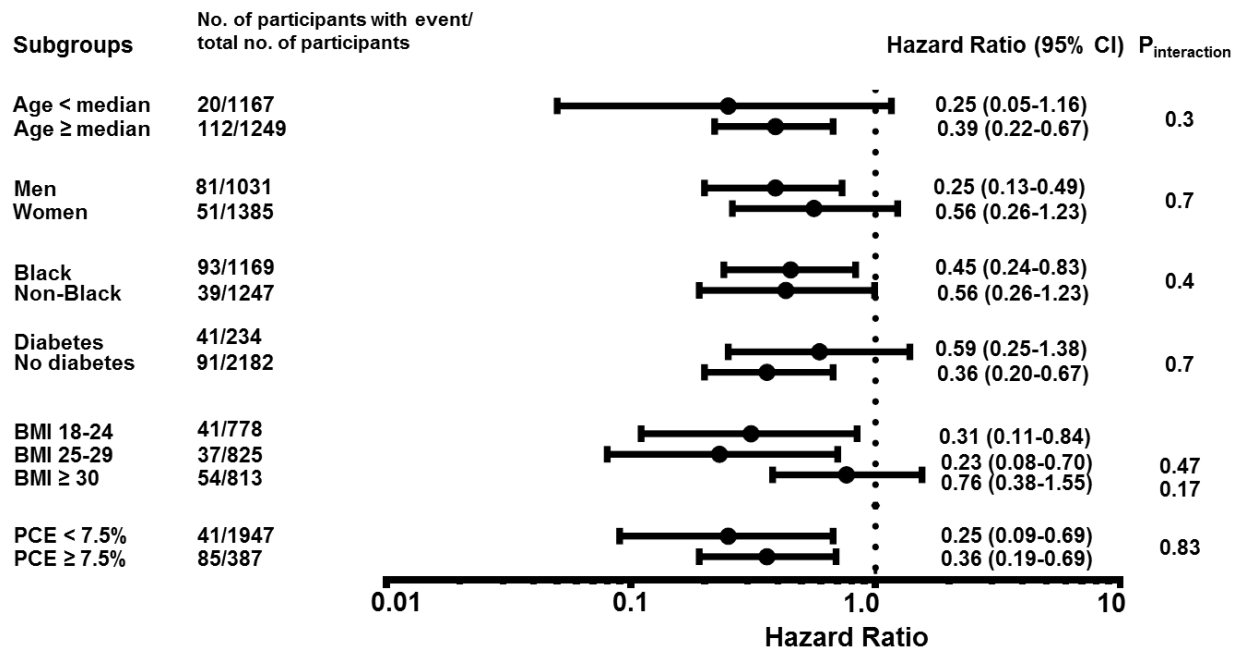
Hazard ratios and 95% confidence intervals (CIs) are shown for increasing quartiles of cholesterol efflux capacity relative to quartile 1. Atherosclerotic cardiovascular disease (ASCVD) events (N=132) include myocardial infarction, stroke, coronary revascularization, and cardiovascular death. Hard atherosclerotic cardiovascular disease events (N=84) include myocardial infarction, stroke, death from myocardial infarction or stroke. Models were adjusted for age, sex, ethnicity, diabetes, hypertension, current smoking, body mass index, total cholesterol, log triglycerides, statin use, HDL cholesterol (HDL-C), and HDL particle number (HDL-P).



### Supplementary Figure S5

#### Unadjusted Hazard Ratios for Cholesterol Efflux Capacity and Incident Cardiovascular Disease by Subgroups.

Hazard ratios and 95% confidence intervals (CIs) are shown for quartile 4 vs 1 for cholesterol efflux capacity for atherosclerotic cardiovascular disease events (N=132) stratified by subgroups. BMI=body mass index. PCE=Pooled Cohort Equation.



**Supplementary Table S1.**

**HDL Cholesterol, HDL Particles, and Cholesterol Efflux Capacity by Sex and Ethnicity**

|           | <b>N</b> | <b>HDL-C<br/>(mg/dL)</b> | <b>HDL-P<br/>(nmol/L)</b> | <b>CEC<br/>(Normalized)<sup>†</sup></b> |
|-----------|----------|--------------------------|---------------------------|---|
| Women     | 1657     | 51 [43-61]               | 33.6 [29.6-38.3]          | 0.99 [0.83-1.18]                        |
| Men       | 1267     | 44 [37-52]*              | 31.6 [28.2-35.4]*         | 1.01 [0.84-1.20]                        |
| Black     | 1443     | 49 [42-60]               | 32.7 [28.7-37.2]          | 0.98 [0.81-1.18]                        |
| Non-Black | 1481     | 45 [38-55]**             | 32.8 [28.9-36.8]          | 1.01 [0.85-1.19]***                     |

Values are medians with interquartile ranges.

HDL-C = HDL cholesterol. HDL-P = HDL particle number. CEC = cholesterol efflux capacity.

† CEC is expressed as a ratio of percent cholesterol efflux of the sample to the percent cholesterol efflux of a reference sample.

\*p<0.001 vs. women; \*\*p<0.001 vs. Black; \*\*\*p<0.01 vs. Black.

**Supplementary Table S2.****Correlates of HDL Cholesterol and Cholesterol Efflux Capacity**

| HDL-C                       |                   | Cholesterol Efflux Capacity |                   |
|-----------------------------|-------------------|-----------------------------|-------------------|
| Model R <sup>2</sup> = 0.35 |                   | Model R <sup>2</sup> = 0.03 |                   |
| Variable                    | Std beta estimate | Variable                    | Std beta estimate |
| Total cholesterol           | 0.22              | Total cholesterol           | 0.12              |
| Alcohol intake              | 0.18              | HDL-C                       | 0.09              |
| Age                         | 0.13              | Male sex                    | 0.05              |
| Exercise dose               | 0.08              | Hypertension                | 0.04              |
| Black ethnicity             | 0.07              | Alcohol intake              | 0.03              |
| Smoking                     | -0.06             | Black ethnicity             | -0.07             |
| Waist to hip ratio          | -0.08             |                             |                   |
| Body mass index             | -0.17             |                             |                   |
| Male sex                    | -0.23             |                             |                   |
| Log Triglyceride            | -0.39             |                             |                   |

Standardized beta estimates and model R<sup>2</sup> values derived from linear regression models modeling continuous measures of HDL cholesterol (HDL-C) and cholesterol efflux capacity. Variables were selected using forward selection with criteria for entry and removal set at p=0.15 and are listed in order of magnitude from positive to negative association. Standardized beta estimates are in standard deviation units of change per 1 standard deviation change of the variable. Model R<sup>2</sup> reflects the total contribution of the model to the variance in the dependent variable (range 0.0-1.0).

**Supplementary Table S3.****Continuous Measurements of Cholesterol Efflux Capacity and Incident First Events**

| Model               | Primary ASCVD<br>(N=132/2416) | Total CVD<br>(N=172/2416) |
|---------------------|-------------------------------|---------------------------|
| Unadjusted          | 0.75 (0.61-0.91)              | 0.84 (0.71-0.98)          |
| TRF-adjusted        | 0.65 (0.53-0.80)              | 0.75 (0.63-0.89)          |
| TRF + HDL-C         | 0.66 (0.54-0.81)              | 0.77 (0.64-0.91)          |
| TRF + HDL-P         | 0.69 (0.56-0.84)              | 0.79 (0.67-0.94)          |
| TRF + HDL-P + HDL-C | 0.68 (0.55-0.84)              | 0.79 (0.67-0.94)          |

Hazard ratios and 95% confidence intervals (CIs) for 1 standard deviation (SD) increase in cholesterol efflux capacity for listed endpoints obtained from Cox proportional hazards models adjusted for traditional risk factors (TRF: age, sex, ethnicity, diabetes, hypertension, current smoking, body mass index, total cholesterol, log triglycerides, statin use), HDL cholesterol (HDL-C), and HDL particle number (HDL-P). Atherosclerotic cardiovascular disease (ASCVD) events include myocardial infarction, stroke, coronary revascularization, and cardiovascular death. Total cardiovascular disease (CVD) events include ASCVD events plus peripheral revascularization, hospitalization for heart failure, and hospitalization for atrial fibrillation. HDL cholesterol (HDL-C) and HDL particle number (HDL-P) were analyzed as continuous covariates.

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