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Lipids, Lipoproteins, and Cardiovascular Risk Factors

# Direct Estimation of HDL-Mediated Cholesterol Efflux Capacity from Serum

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**BACKGROUND:** HDL-mediated cholesterol efflux capacity (HDL-CEC) is a functional attribute that may have a protective role in atherogenesis. However, the estimation of HDL-CEC is based on in vitro cell assays that are laborious and hamper large-scale phenotyping.

**METHODS:** Here, we present a cost-effective high-throughput nuclear magnetic resonance (NMR) spectroscopy method to estimate HDL-CEC directly from serum. We applied the new method in a population-based study of 7603 individuals including 574 who developed incident coronary heart disease (CHD) during 15 years of follow-up, making this the largest quantitative study for HDL-CEC.

**RESULTS:** As estimated by NMR-spectroscopy, a 1-SD higher HDL-CEC was associated with a lower risk of incident CHD (hazards ratio, 0.86; 95%CI, 0.79–0.93, adjusted for traditional risk factors and HDL-C). These findings are consistent with published associations based on in vitro cell assays.

**CONCLUSIONS:** These corroborative large-scale findings provide further support for a potential protective role of HDL-CEC in CHD and substantiate this new method and its future applications.



Circulating HDL particles mediate reverse cholesterol transport by carrying excess cholesterol from the periphery, such as the arterial wall, to the liver for excretion into the bile. HDL cholesterol (HDL-C)[14] is an established epidemiological risk factor for cardiometabolic conditions *(1)*. However, the role of HDL-C remains unclear because most HDL-C increasing therapies have, on the whole, failed to prevent cardiovascular events *(2)*, and Mendelian randomization studies have given consistent evidence that HDL-C is not causal in the development of cardiovascular disease (CVD) *(3)*. Although the recent REVEAL (Randomized EValuation of the Effects of Anacetrapib Through Lipid-modification) trial *(4)* of the cholesteryl ester transfer protein inhibitor anacetrapib resulted in a lower risk of major coronary events, rather than providing evidence for a causal role of HDL-C, these findings were entirely proportional to the reduction in apolipoprotein B-containing lipoproteins *(5, 6)*.

Therefore, the totality of evidence does not support a causal role for HDL-C in coronary heart disease (CHD). This has shifted the focus of HDL research from circulating HDL-C concentrations to other aspects of HDL, such as the functional attributes of HDL particles *(7, 8)*. Cholesterol efflux capacity of HDL (HDL-CEC), which quantifies the ability of HDL particles to extract cholesterol from lipid-laden cells, has emerged as the most widely used metric for HDL function. HDL-CEC reflects the combined action of various HDL particles via multiple cellular pathways *(9)*: intracellular cholesterol is extracted by HDL via adenosine triphosphate (ATP)-binding cassette transporters (ABCA1 and ABCG1), scavenger receptor B1 (SR-B1), and simply by passive

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[14] Nonstandard abbreviations: HDL-C, HDL cholesterol; CVD, cardiovascular disease; CHD, coronary heart disease; HDL-CEC, cholesterol efflux capacity of HDL; ATP, adenosine triphosphate; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; SR-B1, scavenger receptor B1; cAMP, cyclic adenosine monophosphate; ApoA1, apolipoprotein A1; HDL-P, total HDL particle concentration; NMR, nuclear magnetic resonance spectroscopy; HR, hazard ratio: MI, myocardial infarction; IDI, integrated discrimination improvement index; NRI, net reclassification index; RR, risk ratio; Graphic, Genetic Regulation of Arterial Pressure of Humans in the Community; JUPITER, Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin; BODIPY; boron-dipyrromethene.

diffusion *(9)*. There are multiple cellular efflux assays available either to target specific pathways or their combination *(7, 10, 11)*. The most common assay to analyze HDL-CEC uses cyclic adenosine monophosphate (cAMP)-treated J774 murine macrophages with radiolabelled cholesterol *(10, 12–14)*. HDL-CEC measured in cAMP-treated J774 cells incorporates all the aforementioned pathways *(12)*. A fluorescence-labeled cholesterol method has also been used *(11, 14)*. Despite variation in the relative contributions from the different efflux pathways, the correlation between HDL-CEC quantified by these 2 assays is quite high *(11, 15)*.

In recent years, several studies have investigated the association of HDL-CEC with cardiovascular risk in individuals, with quantification of HDL-CEC mainly by cAMP-treated J774 cells *(10, 11, 14, 16)*. These studies were recently summarized in a meta-analysis that strengthened the evidence that HDL-CEC is inversely associated with cardiovascular risk, with the association being independent of HDL-C concentrations *(14)*. However, results from individual studies were inconsistent *(14)* and large-scale evidence on HDL-CEC and cardiovascular outcomes is currently limited to 2 studies, one by Rohatgi et al. *(11)* and the other by Saleheen et al. *(10)*. Both of these studies identified inverse associations between HDL-CEC and cardiovascular events independent of established cardiovascular risk factors, including HDL-C and/or apolipoprotein A1 (apoA1). Interestingly, a recent study suggested HDL-CEC to be heritable, independently of HDL-C *(17)*. In epidemiological studies, HDL-CEC is associated moderately with HDL-related parameters, such as HDL-C *(10, 11, 17)*, HDL size *(11, 17)*, and total HDL particle concentrations (HDL-P) *(11, 17)*, but weakly with clinical variables [such as body mass index (BMI) and blood pressure] *(10, 11)*. Large-scale characterization of the associations of HDL-CEC with multiple cardiometabolic risk factors, as well as HDL subclasses, is currently lacking. The relative paucity of large-scale epidemiology is most likely owing to the complexity and cost of cellular HDL-CEC assays. Novel approaches are needed to facilitate such measurements and enable large-scale investigations of the epidemiological role, genetic architecture, and potential causality of HDL-CEC.

To this end, we have developed a high-throughput, cost-effective alternative approach to the estimation of HDL-CEC through serum nuclear magnetic resonance (NMR) spectroscopy. Recent advancements in experimentation and automated molecular quantifications have made applications of quantitative NMR in epidemiology and genetics increasingly common in recent years *(18, 19)*. These advances have taken NMR-based approaches into large-scale research beyond their well-known role in detailed quantification of lipoprotein subclasses, particles, and lipids *(18–20)*. We show here that it is possible to estimate HDL-CEC from serum NMR spectra and that quantification recapitulates the characteristics of in vitro HDL-CEC in cAMP-treated J774 cells. This report presents the new high-throughput methodology and confirmatory results regarding the associations of HDL-CEC and incident CHD in a large-scale prospective epidemiological study.

## Materials and Methods

An overview of the study is described in Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue8.

### TRAINING DATA

Random blood samples were collected during 2016 from the Finnish Red Cross blood service in accordance with the ethical guidelines required by the Helsinki Declaration. Serum was obtained by centrifugation at 1500*g* for 15 min at ambient temperature and stored at −80 °C. HDL-CEC was measured using cAMP-treated J774 cells within a year of sample collection (details of in vitro HDL-CEC measurement are given in Methods in the online Data Supplement and Table 1 in the online Data Supplement). The same serum samples were also analyzed by proton NMR spectroscopy within the same time frame. The complete training data set obtained comprised 199 individuals with the serum NMR spectra and the corresponding cellular in vitro HDL-CEC estimates. Bayesian regression modeling was applied to link the NMR spectra to the HDL-CEC estimates *(21)*; the correspondence between the NMR-based and the cellular in vitro HDL-CEC estimates is shown in Fig. 2 in the online Data Supplement. Characteristics of the individuals in the training data set are given in Table 2 in the online Data Supplement.

### NMR SPECTROSCOPY AND SAMPLE PREPARATION

A high-throughput NMR spectroscopy platform with an optimized measurement protocol was used to provide quantitative information on the multiple molecular constituents of serum. The experimental details have been previously published *(22)*. The NMR spectroscopic details are identical to the main quantitative NMR metabolomics method *(18, 19)*, and the overall spectral characteristics have been detailed and discussed previously *(18, 22)*. Lipoprotein quantification and HDL-CEC modeling methods are described in Methods in the online Data Supplement.

### EPIDEMIOLOGICAL STUDY POPULATION AND STATISTICAL ANALYSES

The FINRISK97 survey was carried out to monitor the health of the Finnish population among persons of age 25–74 years at recruitment *(23)*. The study was conducted in 5 study areas across Finland, recruiting a total of 8444 persons. The Ethics Committee of the National

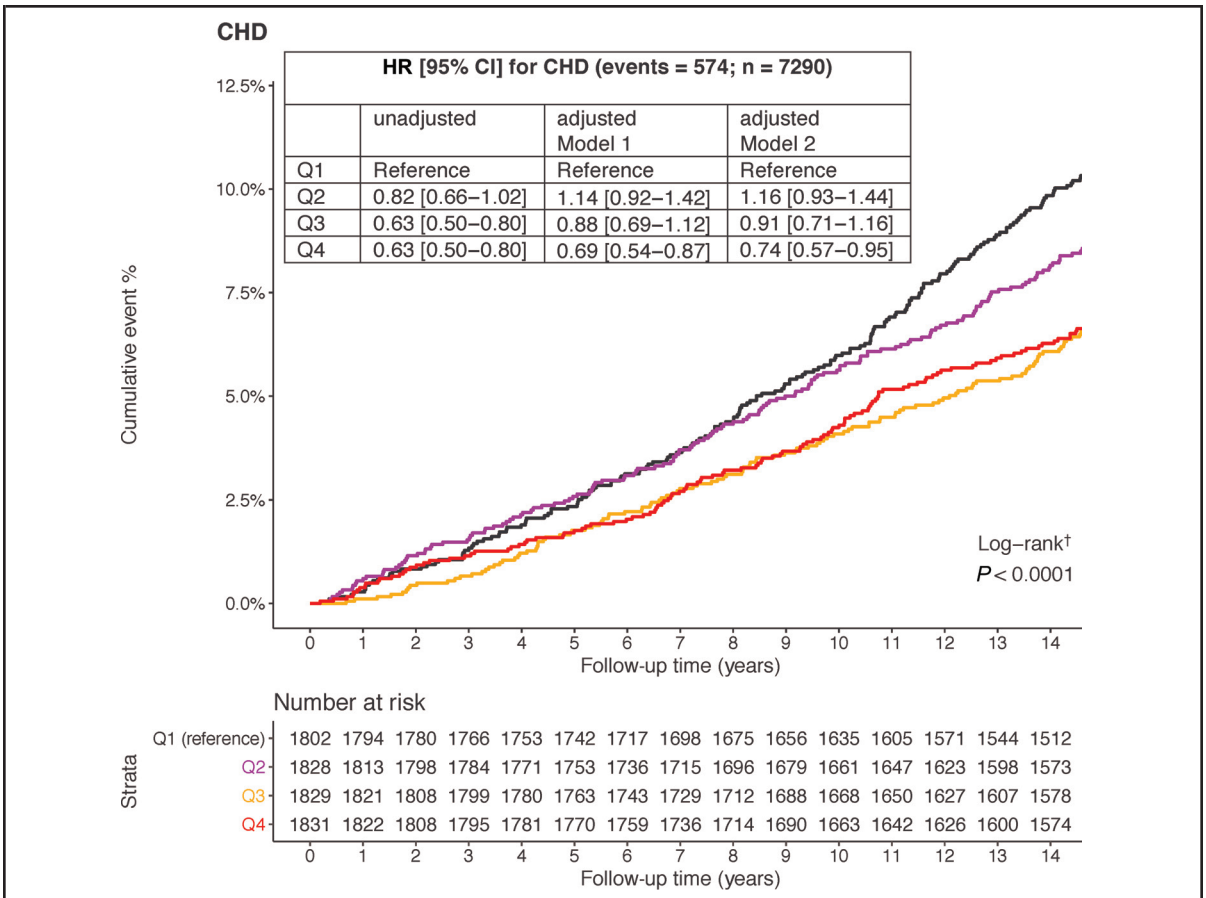


**Fig. 1. Kaplan–Meier cumulative incidence and HRs of CHD by quartiles of NMR-based HDL-CEC.**

HRs were calculated by Cox proportional hazard models with the lowest quartile as the reference group. Model 1: Traditional risk factors [age, sex, geographical region, diabetes, mean arterial blood pressure, blood pressure treatment, smoking, log BMI, total cholesterol, log TG (triglycerides), lipid lowering treatment], HDL-C. Model 2: Model 1, apoA1, and HDL-P. †, Additional statistics for Log-rank test (chi-squared = 22.2; df = 3; 2-sided).

Public Health Institute, Helsinki, Finland, has approved the study in accordance with the Declaration of Helsinki, and written informed consent has been obtained from all participants. Serum samples were collected in 1997 in the semifasting state (median fasting time, 5 h; interquartile range, 4–6 h) and stored at −70°C. The NMR analyses took place in 2012. Statistical analyses are described in Methods in the online Data Supplement.

## Results

### HDL-CEC ESTIMATION VIA SERUM NMR SPECTROSCOPY

Using a Bayesian linear regression model, we established a quantitative relationship between the NMR spectral regions of lipoprotein resonances and in vitro measured HDL-CEC. We found a good correspondence ($R^2$ of 0.83; see Fig. 2 in the online Data Supplement) and low mean bias (see Bland–Altman plot in Fig. 3 in the online Data Supplement) between NMR-based HDL-CEC and in vitro HDL-CEC.

### ASSOCIATION OF HDL-CEC WITH INCIDENT CHD AND CVD EVENTS

Several studies have found inverse associations between HDL-CEC and cardiovascular outcomes, although results are heterogeneous. We were interested to see whether the analysis using NMR-quantified HDL-CEC would replicate findings by prior studies based on in vitro HDL-CEC assays. We analyzed the association of NMR-based HDL-CEC with incident cardiovascular events in a large-scale population-based FINRISK97 cohort (7290 individuals with 574 incident CHD events and 7231 individuals with 789 incident CVD events during 15 years of follow-up; see Methods in the online Data Sup-

plement). The Kaplan–Meier curves in Fig. 1 illustrate the association of NMR-based HDL-CEC quartiles with risk of incident CHD events during follow-up for the individuals in the FINRISK97 cohort. The hazard ratio (HR) between the top and bottom quartile of the NMR-based HDL-CEC values was 0.63 (95% CI 0.50–0.80) (Fig. 1). The event curves show a dose–response at the median threshold, but the association is nonlinear through the quartiles, as reflected also by the cubic spline of the continuous HR across HDL-CEC values (see Fig. 4 in the online Data Supplement). Detailed analyses with multiple adjustments are presented in Table 3 in the online Data Supplement. A similar association was obtained for the association of HDL-CEC with CVD (see Fig. 5 in the online Data Supplement). HDL-CEC remained associated with CHD and CVD events even after adjustment for traditional risk factors and all other HDL-related measures, including HDL-C, apoA1, and total HDL-P; top vs bottom quartile HR for CHD 0.74 (95% CI, 0.57–0.95) (Fig. 1) and HR for CVD 0.79 (95% CI, 0.64–0.98; see Fig. 5 in the online Data Supplement). The trend to an association for hard atherosclerotic CVD, together with myocardial infarction (MI) and stroke separately, was consistent with the association for total CHD (see Table 4 in the online Data Supplement). We also analyzed the association of HDL-CEC for incident CHD events across various clinical subgroups (see Fig. 6 in the online Data Supplement) and detected no subgroup interactions.

Previously Rohatgi et al. *(11)* examined MI and CVD in 2416 individuals over a median follow-up of 9.4 years in a population-cohort with 30 and 172 endpoints, respectively, and Saleheen et al. *(10)* used a prospective nested case–control study with 1745 patients with incident CHD and 1749 control participants. We chose these largest HDL-CEC endpoint studies as a reference. Table 5 in the online Data Supplement presents comparisons for the associations of the in vitro measured HDL-CEC in cAMP-treated J774 cells with MI and CVD *(11)* and CHD *(10)* outcomes with the current results for the NMR-based HDL-CEC estimates and CHD in the FINRISK97 cohort. All the main associations based on the in vitro estimates, including those with various adjustments, were replicated with the NMR-based estimates (see Methods in the online Data Supplement for statistical analyses). Consistent with the findings by Rohatgi et al. *(11)* and Saleheen et al. *(10)* our independent large-scale study also supports the inverse association of HDL-CEC for cardiovascular outcomes. An additional consistent feature in the prior studies together and in our data is that the associations of HDL-CEC with vascular outcomes are robust to multiple adjustments, including HDL-C, apoA1, and total HDL particle concentration (HDL-P).

Rohatgi et al. *(11)* also analyzed the improvement in risk prediction after adding HDL-CEC with traditional risk factors in prediction models. This resulted in small improvements in all the risk-prediction indexes for the primary endpoint (consisting of atherosclerotic CVD), including changes in the *c*-statistic from 0.827–0.841 ($P = 0.02$), the integrated discrimination improvement index (IDI) of 0.02 ($P < 0.001$), and the net reclassification index (NRI) of 0.37 (95% CI, 0.18, 0.56). In our analyses with well-calibrated models (see Fig. 7 in the online Data Supplement), addition of HDL-CEC to traditional risk factors and HDL-C was also associated with small improvements in CHD prediction with improvements in the *c*-statistic [from 0.841 (95%CI, 0.829, 0.854) to 0.843 (95%CI, 0.830, 0.856); $P = 0.02$ by Student *t*-test for dependent samples: *t*.stat = 1.20, df = 7290, 1-sided], IDI of 0.005 (95%CI, 0.001, 0.015), and continuous NRI of 0.21 (95%CI, 0.05, 0.28). Similar improvements were observed for CVD prediction with *c*-statistics [from 0.837 (95%CI, 0.825, 0.848) to 0.838 (95%CI, 0.826, 0.849); $P = 0.01$ by Student *t*-test for dependent samples: *t*.stat = 2.29, df = 7230, 1-sided], IDI of 0.004 (95%CI, 0.001, 0.010), and continuous NRI of 0.18 (95%CI, 0.04, 0.26). Hosmer–Lemeshow statistics for model calibration with CVD were $\chi^2 = 0.021$, $P = 1$, df = 8 and $\chi^2 = 0.020$, $P = 1$, df = 8 for models with and without HDL-CEC, respectively.

We also wanted to compare how the associations for our FINRISK97 cohort using the NMR-based HDL-CEC estimates compared with those from previous studies, as reported in a recent meta-analysis *(14)*. Fig. 2 presents the association of HDL-CEC with cardiovascular risk in this study with comparable data from the meta-analysis *(14)*. The results in the FINRISK97 study were remarkably similar to the summary estimates of the meta-analysis. For example, the risk ratio (RR) estimate for the highest vs lowest quartile of HDL-CEC was 0.69 (95%CI: 0.54, 0.87; Fig. 1) in the FINRISK97 cohort, consistent with the meta-analyzed RR estimate of 0.58 (0.39, 0.86) *(14)*. Similarly, the per 1-SD higher HDL-CEC RR estimate of 0.86 (0.79, 0.93; see Table 3 in the online Data Supplement) in the FINRISK97 cohort was nearly identical to the summary meta-analysis RR of 0.87 (0.73, 1.04) *(14)*. For both of these, there was no evidence of heterogeneity between the current HDL-CEC estimates and the pooled estimates from the meta-analysis (*P* value 0.46 and 0.91 for highest vs lowest and per-1-SD higher, respectively).

### CORRELATIONS OF HDL-CEC WITH VARIOUS ANTHROPOMETRIC, LIPOPROTEIN, AND CLINICAL VARIABLES

The cross-sectional correlations of NMR-based HDL-CEC estimates with various traits in the FINRISK97 cohort are presented in Fig. 3 (exact correlation coeffi-

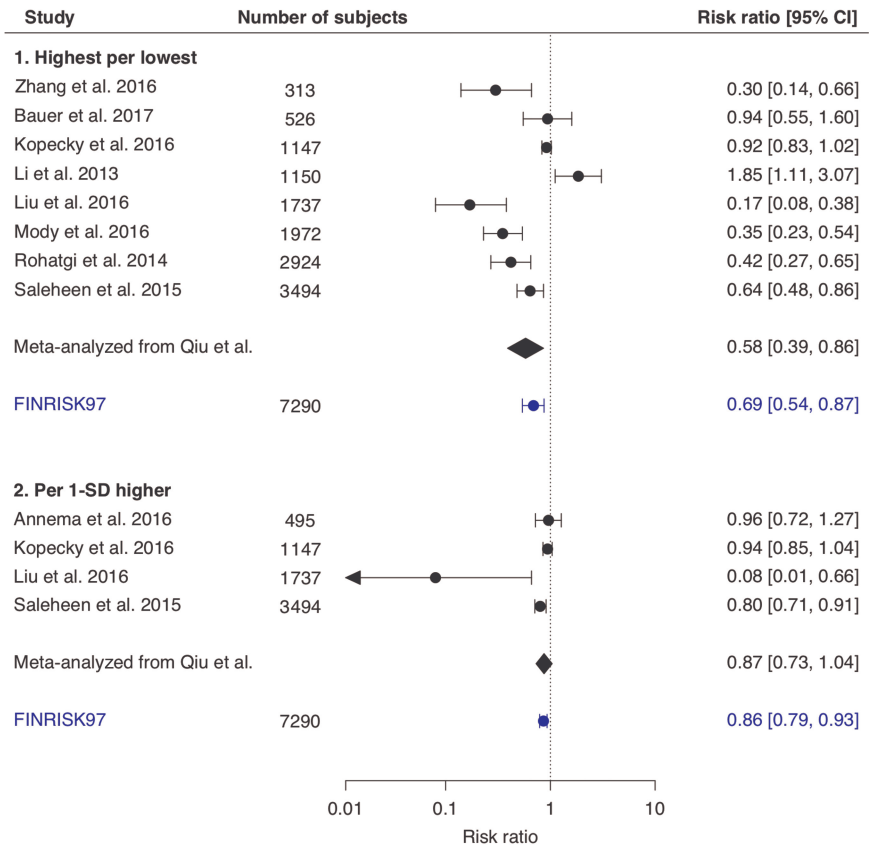


**Fig. 2. Associations of HDL-CEC with cardiovascular risk in the FINRISK97 (NMR-based) compared to previous studies (in vitro cellular assays).**

Previous studies, RR, and their meta-analyzed results were taken from a recent meta-analysis *(14)* investigating the association of HDL-CEC with cardiovascular outcomes. Highest vs lowest denote the RR for CV events comparing the highest to the lowest HDL-CEC quantiles defined in each study. RR from the current FINRISK97 cohort was adjusted for age, sex, geographical region, diabetes, mean arterial blood pressure, blood pressure treatment, smoking, log BMI, total cholesterol, log TG (triglycerides), lipid lowering treatment and HDL-C. Highest vs lowest refers to top vs bottom quartile. There was no evidence of heterogeneity between the current study estimate and the pooled estimate from the meta-analysis by Cochran's Q test, 2-sided ($P = 0.46$; Q = 0.54; df = 1) and ($P = 0.91$; Q = 0.01; df = 1) for highest vs lowest and per-1-SD higher, respectively.

cients are given in Table 6 in the online Data Supplement) together with the corresponding values from Rohatgi et al. *(11)* and Saleheen et al. *(10)*. In addition, Table 7 in the online Data Supplement describes the various traits according to HDL-CEC quartiles. Analogous to the previous studies, we identified positive correlations of HDL-CEC with, for example, HDL-C, HDL particle size, age, blood pressure, alcohol consumption, and female sex. Negative correlations of HDL-CEC were identified for apolipoprotein B, triglycerides, and measures of adiposity. The magnitudes of these correlations were weak, which correspond with the findings reported by Saleheen et al. *(10)*. Unlike Saleheen, we did not find a negative association between diabetes and HDL-CEC. The correlations of HDL-CEC with HDL-C and HDL particle size were even weaker in Rohatgi et al. *(11)*. Although HDL-CEC was positively correlated with HDL-C and other HDL-related measures, the highest correlation of HDL-CEC with HDL-C across these 3 studies was 0.4 (see Table 6 in the online Data Supplement), supporting the view that HDL-CEC and HDL-C contain largely independent information on HDL metabolism.

### ASSOCIATION OF HDL-CEC WITH HDL SUBCLASSES

Serum NMR spectroscopy enables the extensive quantification of lipoprotein subclasses, including particle concentrations for 4 HDL subclasses *(18, 19)*. These data

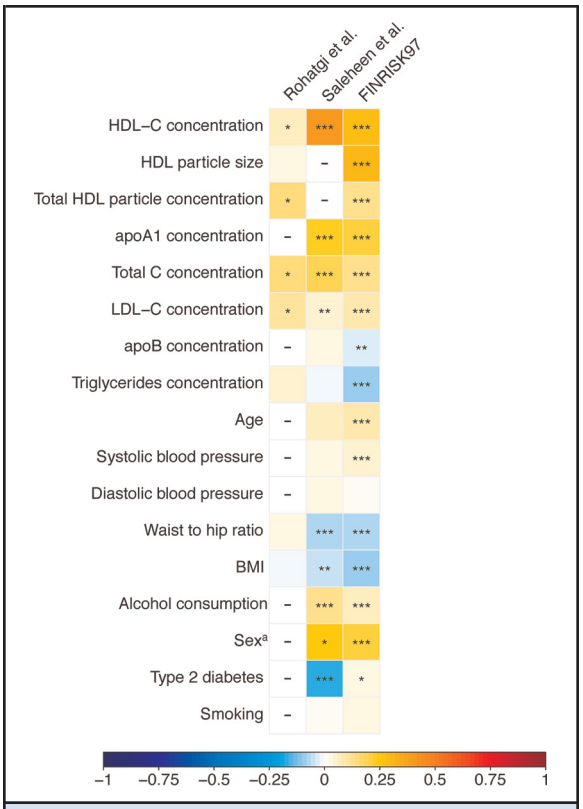


**Fig. 3. Associations of HDL-CEC with clinical and lipid parameters.**

In Rohatgi et al. *(11)* data are Spearman correlation coefficients and in Saleheen et al. *(10)* data are Pearson correlation coefficients adjusted for age and sex. In the FINRISK97 data are Spearman correlation coefficients adjusted for age and sex; n = 7370 (exact correlation coefficients and details in Table 6 in the online Data Supplement). Dash denotes that a correlation coefficient was not available. [a] association for female sex. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

were not available in prior studies including Rohatgi et al. *(11)* or Saleheen et al. *(10)*. Fig. 4 shows correlations between the particle concentrations of 4 HDL subclasses (extra-large, large, medium, and small) and the HDL-CEC, HDL-C, apoA1, and total HDL-P in FINRISK97 (NMR HDL-CEC) and training data (in vitro HDL-CEC). Corresponding correlations stratified by sex are presented in Fig. 8 in the online Data Supplement. HDL-CEC correlated strongly with larger HDL subspecies and the associations between HDL subclass particle concentrations, as well as with other key HDL-related measures, were coherent between the independent data sets. This consistency of association is reassuring because HDL-CEC has been reported to have quite variable associations with HDL-C in different cohorts: the correlation between HDL-CEC and HDL-C was 0.4 in the EPIC (European Prospective Investigation of Cancer)-Norfolk study *(10)*, 0.62 in the Genetic Regulation of Arterial Pressure of Humans in the Community (GRAPHIC) cohort *(17)*, and 0.39 in the Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial data *(13)*. More detailed association analysis between the NMR-based HDL-CEC estimates and HDL subclass lipids is shown in Fig. 9 in the online Data Supplement. The distribution of HDL-CEC values in the FINRISK97 cohort is shown in Fig. 10 in the online Data Supplement and Spearman correlations between the NMR spectral profile and various key HDL-related measures in the training data set are illustrated in Fig. 11 in the online Data Supplement.

## Discussion

We developed a high-throughput method to estimate HDL-CEC directly from serum samples using NMR spectroscopy. The method is based on HDL-CEC estimates from in vitro measurements in cAMP-treated J774 cells, the most common technique to analyze HDL-CEC in cardiometabolic research. The NMR-based HDL-CEC estimates appear to capture the same key aspects as the in vitro HDL-CEC with respect to associations with various anthropometric, lipoprotein, and clinical variables as well as, importantly, with cardiovascular outcomes. We applied the new NMR-based method to estimate HDL-CEC in a large-scale epidemiological study, the FINRISK97 cohort of approximately 7200 individuals, among whom 574 CHD and 789 CVD incident events occurred during 15 years of follow-up. This is currently the largest epidemiological study of HDL-CEC, the results of which also show independent inverse associations of HDL-CEC with risk of CHD and CVD lending support to a potential atheroprotective role of HDL function.

The associations of NMR-based HDL-CEC with risk of cardiovascular outcomes were in keeping with summary estimates of a recent meta-analysis investigating the associations of cellular in vitro HDL-CEC estimates with cardiovascular outcomes *(14)*. A key finding with the NMR-based HDL-CEC corroborating the earlier findings with the cellular in vitro HDL-CEC in the existing largest outcome studies *(10, 11)* was its association with CHD being independent of other HDL-related measures, including HDL-C, apoA1, and HDL-P. Therefore, the NMR-based HDL-CEC estimates recapitulate the characteristics of cellular in vitro HDL-CEC estimates and account for independent information on CHD risk not captured by other HDL-related measures.

Cardiovascular risk prediction is rarely improved by new biomarkers *(24)*. Currently, the ability of HDL-CEC to improve CVD risk prediction beyond traditional

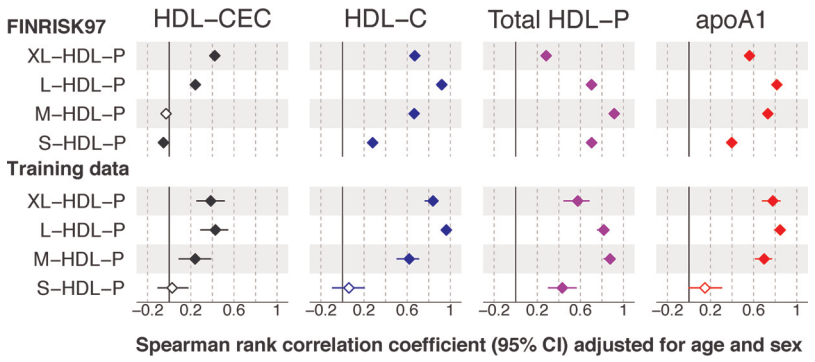


**Fig. 4. Associations of HDL-CEC and related measures with HDL subclass particle concentrations in the FINRISK97 cohort (n = 7597) and in the training data (n = 198).**

The FINRISK97 data are NMR-based HDL-CEC estimates and the training data are from in vitro HDL-CEC measurements. Data are Spearman correlation coefficients (95% CI) adjusted for age and sex. Filled symbols refer to $P < 0.007$ and closed symbols to $P \geq 0.007$. HDL subclasses were measured by NMR spectroscopy and are defined by particle size as follows: XL-HDL, very large (average particle diameter 14.3 nm); L-HDL, large (12.1 nm); M-HDL, medium (10.9 nm); S-HDL, small HDL (8.7 nm) *(19)*. *P* value was adjusted for multiple testing by using principal component analysis (see Methods in the online Data Supplement). HDL-P; total HDL particle concentration (a sum of the individual HDL subclass particle concentrations).

risk factors has been investigated in 2 studies, both describing moderate increases in NRI: 38% *(16)* and 22% *(11)*, but relatively small increases in *c*-statistics. Accordingly, we found an increase in NRI of 21% and small increases in the *c*-statistics. A small increase in *c*-statistic is expected, because this metric is insensitive in model comparisons when good predictors are already present in the reference model *(25)*.

HDL-CEC is a functional measure related to multiple characteristics of HDL particles, and therefore, some level of correlation with other HDL-related measures would be expected. In the FINRISK97 cohort, relatively weak correlations were observed, with the largest being between the NMR-based HDL-CEC estimate and mean HDL-particle size (correlation coefficient, 0.31). In Saleheen et al. *(10)*, the highest correlation was 0.4 between the cellular in vitro HDL-CEC estimate and HDL-C. In the GRAPHIC cohort with HDL-CEC data in 1988 individuals, a correlation of 0.62 between HDL-C and HDL-CEC was found *(17)*, and in the JUPITER trial data, the highest correlation was 0.48 between HDL-CEC and apoA1 *(13)*. Together with the independent associations of HDL-CEC with the risk of vascular disease, this points toward HDL-CEC containing independent information on HDL metabolism and reverse cholesterol transport.

The present study is the first large-scale study investigating the association of HDL-CEC with HDL subclass measures. The HDL-CEC estimates associated with very large and large, but not with medium and small, HDL subclass particle concentrations. These findings match those of previous small-scale studies using cAMP-treated J774 cells *(26, 27)*, and the predominance of the associations with larger HDL particles is most likely due to these particles having a larger receiving area for the diffusing cholesterol molecules and thereby more effective mediation of diffusion than smaller particles *(9)*. This is particularly relevant here because diffusion is thought to be the dominating mechanism for the cholesterol efflux in radioactive cholesterol-labeled cAMP-treated J774 cells *(28)* (see Methods in the online Data Supplement). Diffusion is the main efflux mechanism in cholesterol normal (nonloaded) cells *(28, 29)*, indicating that it has a role in maintaining cholesterol homeostasis in cells at basal conditions, whereas macrophages with cholesterol-loaded states, also known as foam cells, have increased expression of ABC-transporters and enhanced cholesterol efflux through these pathways and decreased contribution of aqueous diffusion-driven pathway *(28, 29)*. Currently, we do not know which pathways are most important in relation to CVD risk *(30)*.

The new cost-effective NMR-based method presented here to estimate HDL-CEC directly from serum samples is designed to correspond to the most common assay to analyze HDL-CEC, i.e., using cAMP-treated J774 murine macrophages with radiolabeled cholesterol *(10, 12–14)*. This latter methodology was used by Saleheen et al. *(10)*, a large-scale outcome study we compared our current results with. The excellent correspondence of our findings (using the NMR-based HDL-CEC estimates) to those by Saleheen et al. *(10)* (using cAMP-treated J774 murine macrophages) serves to corroborate

our approach and is in part expected, given that our NMR assay was developed from the similar in vitro assay. In contrast, the other large-scale outcome study by Rohatgi et al. *(11)* applied a less common, fluorescence-labeled [boron-dipyrromethene (BODIPY)] cholesterol method in a similar cell model *(11, 15)*. The correlation between HDL-CEC estimates from these 2 cellular in vitro assays is modest *(11, 15)*, and it is therefore expected that the NMR-based HDL-CEC results in the FINRISK97 cohort would also match with the results by Rohatgi et al. *(11)* at some extent. However, the main difference between radiolabeled and BODIPY-labeled cholesterol efflux assays is that BODIBY-method favors cholesterol efflux though ABCA1-transporter *(15)* that mediates efflux to small HDL and lipid-poor pre-$\beta$ particles *(31, 32)*, whereas radiolabeled cholesterol assay associates with large HDL particles *(26, 27)* due to movement of radiolabeled cholesterol through all pathways present in cAMP-treated J774 cells, diffusion having the main contributing pathway. Thus, NMR-based HDL-CEC is a proxy for radiolabeled cholesterol efflux assay performed in cAMP-treated J774 cells and it may not represent other efflux models. Despite that, our data are also consistent with the pooled estimate of a recent meta-analysis that summarizes the associations of in vitro HDL-CEC estimates with cardiovascular outcomes *(14)*, which serves to further corroborate our NMR-quantified HDL-CEC.

Although estimated HDL-CEC values seem to recapitulate the key characteristics of in vitro HDL-CEC, applications of this method to diverse populations, i.e., ethnic subgroups, individuals with extreme lipid values or distinct disease states, should be interpreted with care.

Multiple recent drug trials have indicated that increasing circulating HDL-C concentrations does not lead to a reduction in CVD *(5)*. Mendelian randomization studies also fail to support HDL-C having a causal role in CVD *(3)*. We should therefore remain skeptical about the potential causality of HDL-CEC. Nevertheless, the new cost-effective NMR-based method to estimate HDL-CEC could be advantageous in widening the research of cholesterol efflux to large population-based cohorts and drug trials and to expedite appropriately powered studies in relation to multiple cardiovascular and metabolic outcomes. Large-scale cohorts with HDL-CEC estimates are needed to investigate and replicate the associations of HDL-CEC with clinical outcomes and, more importantly, to study the genetic determinants of cholesterol efflux to perform Mendelian randomization analyses for potential causality. We propose the new NMR-based method as a pragmatic alternative for HDL-CEC estimates from in vitro measurements in cAMP-treated J774 cells, particularly in large-scale epidemiology and genetics. This method appears to have great potential to lower the experimental costs related to HDL-CEC measurements and concomitantly speed up collection of the extensive epidemiological evidence base necessary to ascertain whether this functional HDL phenotype is causal for vascular disease and, thus, whether it provides an opportunity for translational applications.

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**Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

S. Kuusisto and M. Ala-Korpela conceived and designed the study, interpreted the results and wrote the manuscript. S. Kuusisto performed the cellular experiments and the statistical analyses and A.J. Kangas the spectral modeling and bioinformatics. M. Karsikas and M. Tiainen prepared the samples and performed the NMR experiments. M.V. Holmes, P. Ohukainen, and J. Kettunen interpreted the results and edited the manuscript. M. Perola and V. Salomaa provided samples and the phenotype data of FINRISK97. All authors discussed the results and approved the final version of the manuscript. M. Ala-Korpela supervised the study.

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