High-density lipoprotein characteristics and coronary heart disease:

a Mendelian randomization study

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ABSTRACT

Aims: The causal role of high-density lipoproteins (HDL) in coronary artery disease (CAD) has been questioned. We aimed to analyse whether genetically determined quantitative and qualitative HDL characteristics were independently associated with CAD.

Methods and Results: We designed a two-sample multivariate Mendelian randomization study with available genome-wide association summary data. We identified genetic variants associated with HDL cholesterol and apolipoprotein A-I quantity, HDL size, particle levels, and cholesterol content to define our genetic instrumental variables in one sample (Kettunen et al study), and analysed their association with CAD risk in a second sample (CARDIoGRAMplusC4D). We validated these results in two other datasets (METSIM and UK-Biobank). Results showed that genetically determined HDL cholesterol and apolipoprotein A-I levels were not associated with CAD risk. HDL mean diameter (β=0.27 [95%CI=0.19; 0.35]) and the levels of cholesterol transported in very large HDL particles (β=0.29 [95%CI=0.17; 0.40] were directly associated with CAD risk, whereas the concentrations of cholesterol transported in medium-sized HDLs (β=-0.076 [95%CI=-0.10; -0.052]) were inversely related to this risk. These results were validated in the METSIM and UK-Biobank data. Genetic variants linked to high cholesterol content in medium-sized HDLs (located within *LIPC* and *PLTP*) were associated with a greater cholesterol efflux capacity (β=0.094 [95%CI=0.013; 0.18]).

Conclusion: Some HDL characteristics, such as particle size and cholesterol content, are related to CAD risk. This relationship could be mediated by a greater cholesterol efflux capacity and is related to *LIPC* and *PLTP*, novel potential therapeutic targets.

Keywords: high-density lipoprotein, HDL quality, coronary artery disease, Mendelian randomization

INTRODUCTION

The inverse association between high-density lipoprotein cholesterol (HDL-C) levels and the risk of coronary artery disease (CAD) has been reported in observational studies[1]. However, experimental and genetic studies question the causality of this association. On the one hand, drugs such as fibrates, niacin, and cholesteryl ester transfer protein inhibitors increase HDL-C levels but do not decrease CAD risk[2]. On the other hand, genetic predisposition to high HDL-C levels has not been linked to any decrease in the risk of cardiovascular events[3,4]. Thus, researchers are looking beyond HDL-C levels to disentangle this apparent contradiction. Anti-atherogenic properties of HDL particles seem to be determined by the quality or function of the lipoprotein[5]. HDL particle size and number were linked to cardiovascular risk[6], and this association could be mediated through HDL functionality, which is predictive of cardiovascular risk[7]. Further evidence of causal association between HDL characteristics and CAD risk would provide relevant data on the validity of these particles as therapeutic targets.

Mendelian Randomization (MR) studies have arisen as a powerful tool to ascertain the potential causality of the association between a biomarker and a disease[8]. These studies assess the association between the genetically determined lifelong values of a biomarker and the development of a clinical outcome. MR studies have already raised serious doubts on the causal role of quantitative HDL characteristics, such as HDL-C and apolipoprotein A-I (ApoA-I) levels, in CAD[3,9]. However, to date, the association between qualitative HDL characteristics and CAD has not been tested using a MR approach. HDL mean diameter, the distribution of cholesterol across the HDL size subtypes, and the concentration of each size subtype HDL particle are some of these qualitative traits. Additionally, this evaluation must take into account the complexity of lipid metabolism and the potential genetic pleiotropic and confounding effects present in the lipid profile[10].

This study had two aims: 1) to assess the potential causal association of quantitative and qualitative HDL characteristics with CAD risk, using a MR approach; and 2) to explore potential mechanisms explaining the observed associations.

METHODS

Study design and data sources

We designed a two-sample MR study using aggregated summary data[8] from four recently published meta-analyses of genome-wide association studies (GWAS). The main analysis was based on data from Kettunen et al[11] and the CARDIoGRAMplusC4D consortium[12], and the validation analysis used the METSIM[13] and UK Biobank CardioMetabolic Consortium datasets[14]. A more detailed description is available in *Supplemental Materials.*

We centred our analysis on the genetic variants associated with: 1) the main lipid profile traits in serum (HDL-C, LDL-C and triglyceride levels); 2) other measurements of HDL quantity (ApoA-I levels); 3) HDL mean diameter; 4) the quantities of cholesterol transported in small, medium-sized, large and very large HDLs; and 5) the levels of HDL particles according to the previous HDL size subtypes.

Genetic instrumental variables of lipoproteins and HDL functionality

We studied the relationship between genetic instrumental variables (GIVs) associated with HDL qualitative characteristics and CAD risk, with both cholesterol efflux capacity and HDL antioxidant capacity. Both functions were determined following standardized methodologies in a random subsample of 643 participants of the REGICOR population-based cohort[15]. A more detailed description is available in *Supplemental Materials*.

Analysis of rare variants with a predicted loss of function effect and CAD risk

We interrogated the MIGen and CARDIoGRAM exome sequencing summary results[16] to explore the association between rare loss-of-function variants in genes of interest and CAD risk. We used the REVEL method for predicting the pathogenicity of rare missense variants[17], selected those with a score >0.7, and meta-analyzed the effect size of their association with CAD.

Statistical analysis

Genetic variants on lipoprotein characteristics and on CAD risk

Five multivariate models were defined *a priori*. Model 1 included HDL-C, LDL-C, and triglyceride levels. Further modelling added the following parameters: ApoA-I levels (Model 2), HDL mean diameter (Model 3), the cholesterol content in each HDL size subtype (Model 4), and the levels of size subtypes of HDL particles (Model 5). In model 4 and 5, we required for the presence of at least two of the HDL subtypes (small, medium-sized, large, and very large) traits to build the model.

For each model, we applied the Multi-Trait-based Conditional & Joint (mtCOJO analysis[18,19]) to the datasets of lipoprotein traits and CAD risk in both the main and the validation analyses. This method allowed retrieval of GIV effects and standard errors on the corresponding lipoprotein trait and on CAD risk, adjusting for other lipoprotein parameters. The genetic correlation between traits was estimated by linkage disequilibrium (LD) score regressions using all genetic variants.

Mendelian randomization analyses

Based on the adjusted GIV effects and their standard errors, we performed the MR analysis using the Generalized Summary-data-based Mendelian Randomization (GSMR) method[19]. The genetic variants to be considered in the GIVs were selected with the following criteria: strong association with the lipid traits of interest (p-value$<5 \cdot 10^{-8}$), not in LD ($R^2 < 0.01$), and a minor allele frequency≥ 0.05. The 1000 Genome project data (http://www.1000genomes.org/phase-3-structural-variant-dataset)[20] was the population of reference to define LD structure. This method also excluded the variants showing pleiotropy, using the HEIDI-outlier test and setting the significance level at a p-value<0.01. We also used Egger regression to study pleiotropic effects of the selected genetics variants[21]. Statistical significance of the results was corrected for multiple comparisons (p-value$=0.05$/number of traits). A description of the sensitivity and *post-hoc* analyses is available in *Supplemental Materials*.

Analysis of lipoprotein GIVs on HDL functionality

We developed genetic risk scores (GRS) weighted by the effect size of the association between the genetic variants of the corresponding lipoprotein trait. Then, we assessed their associations with HDL functionality using the Spearman correlation.

RESULTS

Mendelian randomization results

Selected genetic variants in the GIVs

We identified GIVs for 12 lipoprotein characteristics: levels of HDL-C, LDL-C, triglycerides, and ApoA-I, and qualitative HDL characteristics in the data published by Kettunen et al. In the METSIM data, we identified GIVs for 6 characteristics. We defined as statistically significant those associations with a *p*-value<$4.2 \cdot 10^{-3}$ (0.05/12). The genetic variants included in each GIV and their unadjusted and adjusted effects are listed in Table S1.

We observed high inverse genetic correlations (correlation coefficient≤-0.50) between triglyceride and HDL-C levels. Conversely, we observed very high direct genetic correlations (correlation coefficient≥0.70) between the cholesterol content in each HDL size subpopulation and HDL-C concentrations, between HDL mean diameter and the level of very large HDL particles, and between ApoA-I and the level of very large HDL particles and their cholesterol content (Figure S1).

Main analysis

In the main analysis (Figure 1), we observed a direct association between CAD risk and genetically determined levels of LDL-C (β=0.26 [95% Confidence Interval [CI]: 0.17; 0.35], *p*-value=$1.3 \cdot 10^{-8}$) and triglycerides (β=0.18 [95%CI: 0.073; 0.29], *p*-value=$1.1 \cdot 10^{-3}$). Conversely, CAD risk was not associated with genetically determined concentrations of HDL-C (β=0.008 [95%CI: -

0.084; 0.10], *p*-value=0.87) or ApoA-I (β=0.046 [95%CI: -0.050; 0.14], *p*-value=0.34; Figures 1 and 2).

In qualitative HDL measurements, the genetically determined HDL mean diameter was directly associated with CAD risk (β=0.27 [95%CI: 0.19; 0.35], *p*-value=$2.2 \cdot 10^{-11}$; Figures 1 and 2). Cholesterol content in very large HDLs was also positively linked to CAD risk (β=0.29 [95%CI: 0.17; 0.40], *p*-value=$8.9 \cdot 10^{-7}$), whereas that transported in medium-sized HDLs was inversely related to this risk (β=-0.076 [95%CI: -0.10; -0.052], *p*-value=$4.6 \cdot 10^{-11}$; Figures 1 and 2). Finally, the genetically determined levels of all subtypes of HDL particles showed an inverse trend towards an association with CAD risk, but only that between very large HDLs and CAD was statistically significant (β=-0.22 [95%CI: -0.32; -0.13], *p*-value=$7.1 \cdot 10^{-6}$) (Figure 1). The effect sizes of all the associations are available in Table S2.

Validation analysis

In the validation analysis, we confirmed the direct association between CAD risk and the genetically determined concentrations of LDL-C (β=0.27 [95%CI: 0.20; 0.33], *p*-value=$3.7 \cdot 10^{-15}$) and triglycerides (β=0.089 [95%CI: 0.028; 0.15], *p*-value=0.004), and the null link between CAD risk and the genetically determined HDL-C levels (β=-0.018 [95%CI: -0.073; 0.037], *p*-value=0.53; Figure S2). We also replicated the results regarding qualitative HDL traits: we observed a direct association of CAD risk with genetically determined HDL mean diameter (β=0.44 [95% CI: 0.32; 0.56], *p*-value=$2.6 \cdot 10^{-12}$) and cholesterol content in large HDLs (β=0.39 [95%CI: 0.30; 0.49], *p*-value=$6.1 \cdot 10^{-16}$), but an inverse link between CAD risk and the genetically determined quantity of cholesterol in medium-sized HDLs (β=-0.43 [95%CI: -0.51; -0.34], *p*-value=$9.7 \cdot 10^{-24}$; Figures 1 and S2).

We could not assess the associations between the levels of HDL particles and CAD due to the lack of at least two GIVs related to these HDL traits in the METSIM study. The effect sizes of all the associations of interest are available in Table S3.

Sensitivity analysis

The Egger regression intercept estimates supported the absence of pleiotropic effects. Moreover, the results of the inverse variance weighted method, median-based, and Egger regression were consistent with the main and validation analyses results (Table S4).

The effects on CAD risk of the GIVs of HDL-C, LDL-C and triglyceride levels identified in Kettunen et al were similar to those obtained from the Global Lipid Genetic Consortium (Table S5).

Post-hoc statistical power estimation

Power estimation for the main analyses ranged from 2% to 100% (Table S6).

Genetic instrumental variables of cholesterol content in medium-sized and very large HDLs and HDL functionality

Considering the differential pattern of association between the genetically determined cholesterol content in medium-sized and very large HDLs and CAD risk in the MR analysis, we analysed their relationship with HDL functions.

The GIVs related to the cholesterol quantity in medium-sized HDLs (rs261338 and rs77617917) were located within *LIPC* (encoding the hepatic lipase) and *PCIF1* (close to the gene encoding the phospholipid transfer protein), respectively. The GRS for the cholesterol quantity in medium-sized HDLs was directly associated with cholesterol efflux capacity values (β=0.094 [95%CI: 0.013; 0.18], *p*-value=0.024). The associations of the individual genetic variants associated with cholesterol content in both medium-sized HDLs and very large HDLs with HDL functionality measurements are shown in Table S7.

Analysis of rare variants with a predicted loss of function effect and CAD risk

Two rare variants in *LIPC* with a REVEL score higher than 0.7 were identified in the MIGen and CARDIoGRAM exome sequencing summary results. The meta-analysis of the effect size of their association with CAD is shown in Table S8 with no conclusive results.

DISCUSSION

Our findings suggest a potential causal relationship between qualitative HDL characteristics and CAD risk, even though HDL-C and ApoA-I levels were not associated with CAD risk. In particular, genetically determined mean HDL size and the distribution of cholesterol across HDL size subpopulations were related to CAD risk. Our results also indicate that the protective effect of small HDLs on CAD risk could be related to greater cholesterol efflux capacity.

The relationship between HDL and cardiovascular risk is controversial[4]. Recent studies suggest that HDL functions, rather than HDL-C concentration, are the main determinants of HDL anti-atherogenic properties[5]. Our data are consistent with previous evidence, and reflect that HDL-C and ApoA-I levels in the bloodstream are not causally related to CAD[3,9]. However, we observed a decrease in CAD risk when HDL-C was mainly transported in smaller HDLs, but an increase in CAD risk when HDL-C was mainly transported in larger particles. These results may explain why HDL-C levels are not causally associated with cardiovascular risk, as the protective effect of cholesterol content in small or medium-sized HDLs may be counterbalanced by the adverse effect of cholesterol content in larger particles. These results are consistent with previous experimental evidence, and could contribute to explain the therapeutic failure of the pharmacological agents known to increase HDL-C levels. Niacin or cholesteryl ester transfer protein inhibitors are effective in increasing HDL-C concentrations but not in reducing CAD risk; this paradox could be explained by a promotion of the accumulation of cholesterol content in large HDLs[2]. In gemfibrozil-treated patients changes in HDL-C levels accounted for a small proportion of the CAD risk reduction (<10%), whereas the increase in small HDLs was much more predictive of this risk reduction[22]. Our results also concur with genetic studies analysing variants in the *SR-B1* gene, showing that individuals with loss-of-function variants have higher HDL-C concentrations, mainly in very large particles, but also higher CAD risk[23].

However, there is still controversy in the relationship between HDL size subtypes and cardiovascular risk: some authors advocate for small HDLs as an indication of lower CAD risk[24] while others suggest they are associated with increased CAD risk[25]. There are several possible explanations for at least part of this heterogeneity. First, the baseline health condition of the subjects affects HDL quality and function. Lipid-poor, protein-rich, small HDLs could be dysfunctional in pro-oxidative and

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pro-inflammatory pathological states due to post-translational modifications of their proteins and their enrichment in pro-inflammatory mediators (such as serum amyloid A or complement 3)[26]. Second, laboratory procedures to measure HDL size (NMR spectroscopy, electrophoresis, etc.) differed between the published studies, and there is low concordance in results from these techniques[27]. Third, the statistical models used did not consider all the same confounding factors and did not always include as covariates the levels of HDL-C or other lipid profile parameters deeply interrelated with these lipoproteins (e.g. triglyceride concentrations).

Our results suggest that the protective effect of small HDLs on CAD risk could be related to greater cholesterol efflux capacity. The top genetic variant associated with higher cholesterol content in medium-sized HDLs, increased cholesterol efflux, and lower CAD risk is located within *LIPC*, which encodes the hepatic lipase C, able to hydrolyse triglycerides in circulating lipoproteins, including HDL particles[28]. Aged HDL particles become triglyceride-rich, a characteristic inversely related to ApoA-I stability in the HDL particle. Greater ApoA-I instability may be related to its lower cholesterol efflux capacity and HDL antioxidant ability and to greater disintegration of the HDL structure[29]. The hydrolysis of HDL triglycerides by hepatic lipase generates small/medium-sized, triglyceride-depleted particles, considered more stable and functional than very large, triglyceride-rich HDLs[28]. The second genetic variant associated with the cholesterol in medium-sized HDLs is found within a locus close to *PLTP* , which encodes the phospholipid transfer protein, involved in HDL remodeling/stabilization and the generation of lipid-free/lipid-poor small HDLs[28]. The association between these two loci and cholesterol efflux capacity has also been observed in a previous GWAS[30]. Therefore, HDL functionality and remodelling through LIPC and PLTP activity could play a key role in this equation. Unfortunately, we could not validate the potential impact of a loss-of-function mutations by interrogating rare variants from exome sequencing studies due to the limited statistical power of these studies (*LIPC*) or the lack of functional rare variants (*PLTP*).

Both observational and experimental studies have more consistently found an inverse relationship between the number of HDL particles and cardiovascular risk, compared to HDL-C levels[7]. Similarly, we observed that the concentrations of HDL particles of all sizes were inversely related to CAD risk, although only the levels of very large HDLs were significantly associated in the

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main analysis. Unfortunately, we could not validate these results due to the lack of valid GIVs in the METSIM study.

Our study has several methodological strengths. First, our results are based in MR, a useful approach to explore the causality of the association between biomarkers and specific diseases. However, some assumptions must be made[8], especially the lack of pleiotropy. In our case, most of the genetic variants used as instruments were associated with more than one lipid trait. To solve this problem, we used a novel approach (mtCOJO-GSMR methodology) to control for the confounding effects related to the close relationship between lipoprotein characteristics. This method also takes into account the uncertainty of the effect size estimation of the associations of the GIVs[18]. Second, we included two independent MR analyses to validate the results initially observed. Finally, the validity of the GIVs for HDL-C, LDL-C and triglyceride levels initially generated was confirmed, supporting the validity of these datasets for other GIVs we generated. However, our study also has limitations. First, the statistical power of our analyses is limited for some of the traits of interest. Second, in the validation analysis we could not generate GIVs for some of the lipoprotein traits.

In conclusion, our study indicates that several genetically and life-long qualitative HDL characteristics were related to CAD risk. Although HDL-C and ApoA-I levels were not causally linked to CAD risk, our results support a potential causality between cholesterol content in very large HDLs and higher CAD risk, and between cholesterol in medium-sized s and lower CAD risk. The protective capacity of medium-sized HDLs could be partially mediated through a greater cholesterol efflux capacity that could be related to the activity of the hepatic lipase and the phospholipid transfer proteins, suggested as potential therapeutic targets for further exploration.

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CONFLICT OF INTEREST

The authors declare they do not have conflict of interest.

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FIGURE LEGENDS

Figure 1. Association of the genetically determined main lipid traits and HDL characteristics with coronary artery disease (CAD) risk in the two Mendelian randomization analyses performed. Figure 1 caption: Effect of HDL cholesterol (HDL-C), LDL cholesterol (LDL-C) and triglyceride levels (Model 1), Apolipoprotein A-I levels (Model 2), mean diameter of the lipoproteins (Model 3), levels of particles of each size subtype (Model 4) and cholesterol content of each HDL size subtype (Model 5) on CAD. The main analysis (Kettunen and CARDIoGRAMplusC4D) is highlighted with orange lines. The validation analysis (METSIM and UK Biobank) is highlighted with grey lines.

Figure 2. Association of individual SNPs with (A) HDL cholesterol (HDL-C) levels, (B) apolipoprotein A1 (ApoA-I) levels, (C) mean HDL particle diameter, (D) cholesterol content in medium-sized HDLs, and (E) cholesterol content in very large HDLs with coronary artery disease risk (CAD). Figure 2 caption: Estimates of the associations were derived from the study by Kettunen et al and from the CARDIoGRAMplusC4D meta-analyses (multivariate adjusted estimates). Error bars represent 95% confidence intervals. The slopes of the lines show the genetic instrumental variable regression estimates of the effect of the lipid characteristics of interest on coronary artery disease risk.

Odds Ratio (xSD)

A. *HDL-C* — SNP-CAD association (y-axis) vs SNP - HDL-C levels association (x-axis)

B. *ApoA-I* — SNP-CAD association (y-axis) vs SNP - ApoA-I levels association (x-axis)

C. *HDL* — SNP-CAD association (y-axis) vs SNP - Mean diameter association (x-axis)

D. *Medium HDL particles* — SNP-CAD association (y-axis) vs SNP - Cholesterol content association (x-axis)

E. *Very large HDL particles* — SNP-CAD association (y-axis) vs SNP - Cholesterol content association (x-axis)