Novel insights into blood markers and cardiovascular disease: Results of the Netherlands Epidemiology of Obesity study

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SECTION II

LIPIDS

CHAPTER 6

MENDELIAN RANDOMISATION ANALYSIS OF CHOLESTERYL ESTER TRANSFER PROTEIN AND SUBCLINICAL ATHEROSCLEROSIS: A POPULATION-BASED STUDY

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Abstract

Background

Several trials to prevent cardiovascular disease by inhibiting cholesteryl ester transfer protein (CETP) have failed, except REVEAL. Thus far, it is unclear to what extent CETP is causally related to measures of atherosclerosis.

Objective

To study the causal relation between genetically-determined CETP concentration and carotid intima media thickness (cIMT) in a population-based cohort study.

Methods

In the Netherlands Epidemiology of Obesity study, participants were genotyped, and cIMT was measured by ultrasonography. We examined the relation between a weighted genetic risk score for CETP concentration, based on 3 SNPs that have previously been shown to largely determine CETP concentration and cIMT using Mendelian randomisation in the total population and in strata by sex, Framingham 10-year risk, (pre)diabetes, HDL-cholesterol, triglycerides and statin use.

Results

We analysed 5,655 participants (56% women) with a mean age of 56 (range 44-66) years, BMI of 26 (range 17-61) kg/m^2 and serum CETP of 2.47 (range 0.68-5.33) µg/mL. There was no evidence for a causal relation between genetically-determined CETP and cIMT in the total population, but associations were differently directed in men (16µm per µg/mL increase in genetically-determined CETP; 95% CI: -8, 39) and women (-8 µm; -25, 9). Genetically-determined CETP appeared to be associated with cIMT in normoglycemic men (26 µm; -1, 52) and in (pre)diabetic women (48 µm; -2, 98).

Conclusion

In this population-based study, there was no causal relation between genetically-determined CETP concentration and cIMT in the total population, although we observed directionally differing effects in men and women. Stratified results suggested associations in individuals with different cardiometabolic risk factor profiles, which require replication.

Introduction

Recent studies to improve cardiovascular risk prevention have focussed on cholesteryl ester transfer protein (CETP) inhibitors since they increase HDL-c and decrease non-HDL-c concentrations. [20, 176, 177]

CETP facilitates the migration of cholesteryl esters from HDL to LDL and very low-density-lipoproteins (VLDL). A high CETP concentration is therefore hypothesized to contribute to an atherogenic lipoprotein profile by increasing (V) LDL-c and decreasing HDL-c.[178] Several observational studies have suggested that lower concentrations of CETP are associated with reduced CVD risk. [179, 180] Most recent efforts to lower CETP concentration pharmacologically with the purpose of reducing CVD risk have been unsuccessful, except for the REVEAL trial, in which CETP inhibition with anacetrapib successfully lowered the risk of major coronary events in high-cardiovascular risk patients. [48, 181] The effect of genetically determined CETP has been subject to considerable discussion in recent literature, but in general a detrimental effect of high CETP, if any, appears to be restricted to men [49, 50, 176, 178, 181-189] Close inspection of previous studies on the association of CETP with CVD risk suggested that in addition to sex, other factors potentially modulate the effects of CETP on CVD risk, including HDL-c or triglyceride concentrations, insulin resistance, or the use of statins or fibrates. [20, 50, 51, 190, 191] This suggests that CETP inhibition could be effective in specific subgroups of the population. To provide more insights in the role of CETP on cardiovascular risk , we aimed to study the causal effect of genetically determined higher CETP concentration on atherosclerosis in the general low-risk population, as well as specific subgroups, using a genetic risk score for CETP concentration as determinant.

Methods

Study design and study population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study of 6,671 men and women aged between 45 and 65 years. The study design and population are described in detail elsewhere.[59] All inhabitants with a self-reported body mass index (BMI) of 27 kg/m^2 or higher and living in the greater area of Leiden, the Netherlands were eligible to participate in the NEO study. In addition, all inhabitants aged between 45 and 65 years from one adjacent municipality (Leiderdorp, the Netherlands) were invited to participate irrespective of their BMI, allowing for a reference distribution of BMI. Participants visited the NEO study centre for extensive baseline measurements, including blood sampling and cIMT. Research nurses recorded current medication use by means of a medication inventory. Prior to the study visit, participants completed questionnaires at home with respect to demographic, lifestyle, and clinical information.

The Medical Ethical Committee of the Leiden University Medical Centre (LUMC) approved the protocol. All participants gave their written informed consent.

For the present analyses, we excluded participants from non-European ancestry or with poor genotyping quality (n=927) : when the sample call rate was <98%, there was a sex mismatch, heterozygosity rate was not within ±3 SD of mean heterozygosity rate, participants differed based on the first two principal components (PCs) (±3.5 SD), samples were duplicates, or concordance with another DNA sample was >0.25 (related individuals). Furthermore, we excluded participants with missing CETP (n=31) and cIMT measurement (n=58).

Blood sampling

During the visit to the NEO study centre, venous blood samples were obtained from the antecubital vein after a >10 hour overnight fast. Fasting serum total cholesterol and TG concentrations were measured with enzymatic colorimetric assays (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany) and fasting serum HDL-c concentrations with third generation homogenous HDL-c methods (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany). LDL-c concentrations were calculated using the Friedewald equation.[128] Furthermore, aliquots of plasma and serum were stored at -80°C after centrifugation. DNA was extracted and genotyping was performed by the Centre National de Génotypage (Evry Cedex, France), using the Illumina HumanCoreExome-24 BeadChip (Illumina Inc., San Diego, California, United States of

America). Subsequently, genotypes were imputed to the 1000 Genome Project reference panel (v3 2011) using IMPUTE (v2.2) software. [104, 105] Three variants within the CETP gene were discovered in a genome wide association study in this population: rs12720922, rs247616 and rs1968905 were used for the present study, of which rs12720922 and rs1968905 were imputed variants (Blauw et al, submitted).

CETP concentrations were measured in serum that had undergone one previous freeze-thaw cycle with enzyme-linked immune sorbent assay (ELISA) kits according to the manufacturer's instructions (DAIICHI CETP ELISA, Daiichi, Tokyo, Japan; coefficient of variation 11.7%).

Subclinical atherosclerosis

Carotid intima media thickness (cIMT) was used as a measure of subclinical atherosclerosis. cIMT was assessed by ultrasonography of the common carotid arteries (CCA). A 15 mm long section 10 mm proximal of the CCA bifurcation was measured while the subject was in supine position. cIMT was measured using a 7.5–10 MHz linear-array probe and the Art.Lab system in B-mode setting and using a wall-track system (ART.LAB version 2.1, Esaote, Maastricht, The Netherlands) to detect boundaries between lumen and intima, as well as between media and adventitia. cIMT was measured during six heart beats in angles of 180, 135 and 90 degrees (right CCA) and 180, 225 and 270 degrees (left CCA). We calculated the mean IMT for each participant by averaging the 36 cIMT measurements within each individual. Measurements of cIMT were validated by analysing repeated measurements in 169 randomly selected participants, which resulted in an intra-observer CV of 5.8% and an inter-observer CV of 9.0%. Detailed results of the validation study are presented in Appendix 1.

Other variables

On the questionnaire, participants reported their highest level of education in ten categories according to the Dutch education system, which was further reclassified into low (none, primary school or lower vocational education) or high (other). Participants reported their medical history of diabetes and cardiovascular diseases. Pre-existing CVD was defined as myocardial infarction, angina pectoris, congestive heart failure, stroke, or peripheral vascular disease. In addition, all use of medication in the month preceding the study visit was recorded. Tobacco smoking was reported in three categories: never smoker, former smoker or current smoker. Participants reported their physical activity during leisure time, which was expressed in metabolic equivalent hours per week. Menopausal state was categorized in pre-, and postmenopausal state according to informa-

tion on ovariectomy, hysterectomy and self-reported state of menopause in the questionnaire. The Framingham 10-year risk of cardiovascular disease was calculated by summation of component scores based on age, LDL-c, HDL-c, diastolic and systolic blood pressure, diabetes, and smoking. [192]

Statistical analysis

The present study is a cross-sectional analysis of the baseline measurements of the NEO study. In the NEO study, individuals with a BMI of 27 kg/m^2 or higher were oversampled. To represent baseline associations in the general population adjustments for the oversampling of individuals with a BMI ≥ 27 kg/m^2 were made.[60] This was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality.[167] The BMI distribution of the participants from Leiderdorp was similar to the BMI distribution of the general Dutch population.[16] All results were based on weighted analyses.

Data were summarized by sex as mean (SD; normally distributed data only), median (25^{th}, 75^{th} percentiles; non-normally distributed data only), or as percentage (categorical data).

We examined the observational association between CETP concentration and cIMT using multivariate linear regression analysis. Results are presented as beta coefficients with 95% confidence intervals (CI) which can be interpreted as difference in cIMT (µm) per unit (µg/mL) of measured CETP concentration. These regression models were adjusted for age and sex, LDL-c concentrations, ethnicity, education, physical activity and smoking. Adjustment for LDL-c was performed to evaluate the association between CETP and IMT through HDL-c. Because it is not completely known whether the following factors are common causes of both CETP and cIMT, and therefore their role as confounding factors is doubted, we performed separate observational analyses additionally adjusted for statin use, diabetes and hypertension.

We calculated a weighted genetic risk score (GRS) by summation of the products of the number of alleles per SNP and the effect size per allele. We estimated the association of the separate SNPs and the GRS with CETP concentrations using linear regression analyses.

We estimated the causal relation between the separate SNPs for CETP concentration and cIMT using linear regression analyses, and we examined the causal association between CETP concentration and cIMT using an instrumental variable two-stage-least-squares regression analysis in which CETP concentration was instrumented by a genetic risk score composed by the three genetic vari-

ants in the *CETP* gene. The regression coefficients from these analyses can be interpreted as difference in cIMT (μm) per unit (μg/mL) in genetically-determined CETP concentration.

The analyses were performed in the total population, and separately for pre-specified subgroups based on sex, high CVD risk profile (defined as a Framingham predicted 10-year risk of 10% or higher), (pre)diabetes (defined as having fasting glucose concentrations ≥6.1 mmol/L, self-reported diabetes or using glucose-lowering medication), high HDL-c concentration (with a cut-off value of 1.04 mmol/L in men, and 1.30 mmol/L in women), triglyceride concentration (with a cut-off value of 1.7 mmol/L), statin use, and menopausal status. [193-195]

Analyses were performed in Stata (version 14, StataCorp. 2015)

Results

Baseline characteristics

Table 1 – Baseline characteristics of the participants of the Netherlands Epidemiology of Obesity study, men and women aged between 45 and 65 years (n=5,655).

	Total population (n=5,655)	CETP ≤ median (2.60 µg/mL)	CETP > median (2.60 µg/mL)
Age (y)	56 (6)	56 (6)	56 (6)
Sex (male)	44	52	36
BMI (kg/m2)	26 (4)	26 (4)	26 (4)
Tobacco smoking (% never)	38	36	41
Physical activity (MET h/week)	30 (16-50)	30 (16-50)	30 (16-50)
Education level (% high)	47	49	46
(Pre)diabetes (%)	14	17	11
Hypertension (%)	34	34	34
CVD (%)	5	7	3
Statin use (%)	10	15	5
Framingham 10-year risk ≥10%	24	24	23
Total cholesterol (mmol/L)	5.7 (1.0)	5.5 (1.0)	5.9 (1.0)
HDL cholesterol (mmol/L)	1.6 (0.5)	1.6 (0.5)	1.6 (0.5)
LDL cholesterol (mmol/L)	3.5 (1.0)	3.3 (0.9)	3.8 (0.9)
Triglycerides (mmol/L)	1.2 (0.9)	1.2 (0.8)	1.2 (0.9)
CETP (µg/mL)	2.47 (0.65)	1.96 (0.32)	2.98 (0.47)
Coding allele rs247616 (%)	56	66	46
Coding allele rs12720922 (%)	17	11	23
Coding allele rs1968905 (%)	82	80	84

Results were based on analyses weighted towards the BMI distribution of the general population (2,715 men and 2,940 women). Results are shown as mean (SD), median (25th, 75th percentiles) or percentage.

BMI, body mass index; CETP, cholesteryl ester transfer protein; cIMT, carotid intima media thickness; HDL, high density lipoprotein; LDL, low density lipoprotein; MET, metabolic equivalent of task; NEO, Netherlands Epidemiology of Obesity.

Baseline characteristics of the study population are presented in Table 1, stratified by sex. The mean (SD) age of the participants was 56 (6) years, and 56% were women. The mean BMI was 26 (4) kg/m^2, mean CETP concentration was 2.47 (0.65) µg/mL, and the mean cIMT was 616 (92) µm.

Serum CETP concentration and atherosclerosis

Table 2 – Associations of observed CETP concentrations (adjusted), CETP SNPs, and genetically-determined CETP concentrations with the intima media thickness in the total population of the Netherlands Epidemiology of Obesity study (n = 5,655) and in men and women separately.

	Difference in cIMT in µm (95% CI)		
cIMT	Total population	Men (44%)	Women (56%)
CETP concentration (per µg/mL) (crude)	-2 (-7, 4)	8 (-1, 17)	-0 (-7, 6)
CETP concentration (per µg/mL) (adjusted)	-1 (-6, 5)	10 (1, 19)	-7 (-14, -1)
CETP concentration (per µg/mL) (additionally adjusted)	-1 (-6, 5)	10 (1, 19)	-7 (-14, -0)
rs1968905-G (per risk-increasing allele)	2 (-5, 9)	-2 (-13, 10)	3 (-7, 12)
rs247616-C (per risk-increasing allele)	0 (-5, 6)	-4 (-12, 4)	4 (-4, 11)
rs12720922-A (per risk-increasing allele)	2 (-5, 8)	7 (-3, 17)	-3 (-11, 5)
Genetically-determined CETP (µg/mL)	2 (-12, 16)	16 (-8, 39)	-8 (-25, 9)

Results were based on analyses weighted towards the BMI distribution of the general population (n=5,655), and were derived from beta coefficients (95% CI) from linear regression and expressed as difference in cIMT µm. Results for the genetic risk score were presented as µm difference in cIMT per µg/mL genetically-determined CETP concentration. Observational analyses were adjusted for age, LDL-c concentration, ethnicity, education, physical activity, and smoking. The additionally adjusted model was additionally adjusted for statin use, diabetes and hypertension. The observational analyses in the total population was additionally adjusted for sex.
CETP, cholesteryl ester transfer protein; cIMT, intima media thickness; LDL-c, low density lipoprotein cholesterol; SNP, single nucleotide polymorphism.

In observational analyses in the total population, CETP concentration was weakly associated with cIMT (adjusted coefficient: -1 µm per µg/mL CETP; 95% CI: -6, 5). Table 2 reports stratified analyses with a weak positive association in men (10 µm per µg/mL higher CETP concentration; 95% CI: 1, 19) and a weak negative association in women (-7 µm per µg/mL CETP; 95% CI: -14, -0)

Associations between genetically-determined CETP and atherosclerosis

The genetic risk score (GRS) of three independent CETP SNP risk alleles rs247616-C, rs12720922-A, and rs1968905-G accounted for 14.7% of variation in CETP concentrations in the participants of the NEO study.

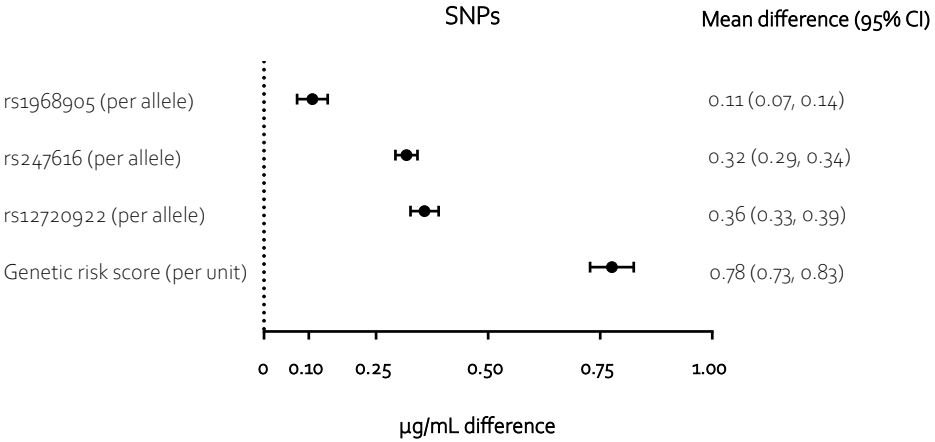


Figure 1 – Associations with 95% confidence intervals of SNPs rs1968905, rs247616, and rs12720922 and the genetic risk score composed from these SNPs with serum CETP concentrations in the participants of the Netherlands Epidemiology of Obesity study, men and women aged between 45 and 65 year (n=5,655). Associations are expressed in µg/mL CETP per allele of the SNPs or per unit of the genetic risk score.

Figure 1 reports the difference in CETP concentration per CETP-increasing SNP allele and per point increase in GRS: 0.78 µg/mL (95% CI: 0.73, 0.83). Table 2 reports causal relations between variations in the *CETP* gene and cIMT. CETP was not causally related to cIMT in the total study population. We observed a causal relation between CETP and cIMT in men of 16 µm (95% CI: -8, 39) difference in cIMT per µg/mL genetically-determined CETP in men and -8 µm (95% CI: -25, 9) in women.

Subgroup analyses

Men and women were stratified for CVD risk profile, (pre)diabetes, fasting HDL-c, triglycerides and statin use. The results of the stratified analyses reported in Figure 2 and Figure 3 demonstrate that in men with a low 10-year Framingham risk, one µg/mL genetically-determined CETP was related to 12 µm (95% CI: -12, 36) thicker cIMT, while in men with a 10-year risk ≥10% the relation was 24 µm (95% CI: -25, 72) per µg/mL genetically-determined CETP. In men without

(pre)diabetes one µg/mL genetically-determined CETP was related with 26 µm (95% CI: -1, 52) thicker cIMT per µg/mL, and with -24 µm (95% CI: -66, 18) in men with (pre)diabetes. In men with normal HDL-c, normal TG concentrations, or not using statins, the associations ranged between 19 and 21 µm per µg/mL genetically-determined CETP concentration, while in men with low HDL-c CETP was inversely related to cIMT (-20 µm per µg/mL CETP; 95% CI: -67, 27).

One µg/mL genetically-determined CETP was related with a -17 µm (95% CI: -36, 1) difference in cIMT in women with a low 10-year Framingham risk, while in women with a 10-year Framingham risk ≥10% this relation was 23 µm (95% CI: -14, 59) per µg/mL genetically-determined CETP. In women with (pre)diabetes, one µg/ml genetically-determined CETP was related with a 48 µm (95% CI: -2, 98) thicker cIMT, and with -13 µm (95% CI: -31, 5) difference in cIMT in women without (pre)diabetes. We observed relations close to the null in women with low HDL-c, high TG concentrations, or who used statins. In women with normal HDL-c, fasting TG, or not using statins, the relations ranged between -9 and -14 µm per µg/mL CETP. In premenopausal women, one µg/mL genetically-determined CETP was related with a -25 µm (95% CI: -59, 8) difference in cIMT, and -0 (-19, 18) in postmenopausal women.

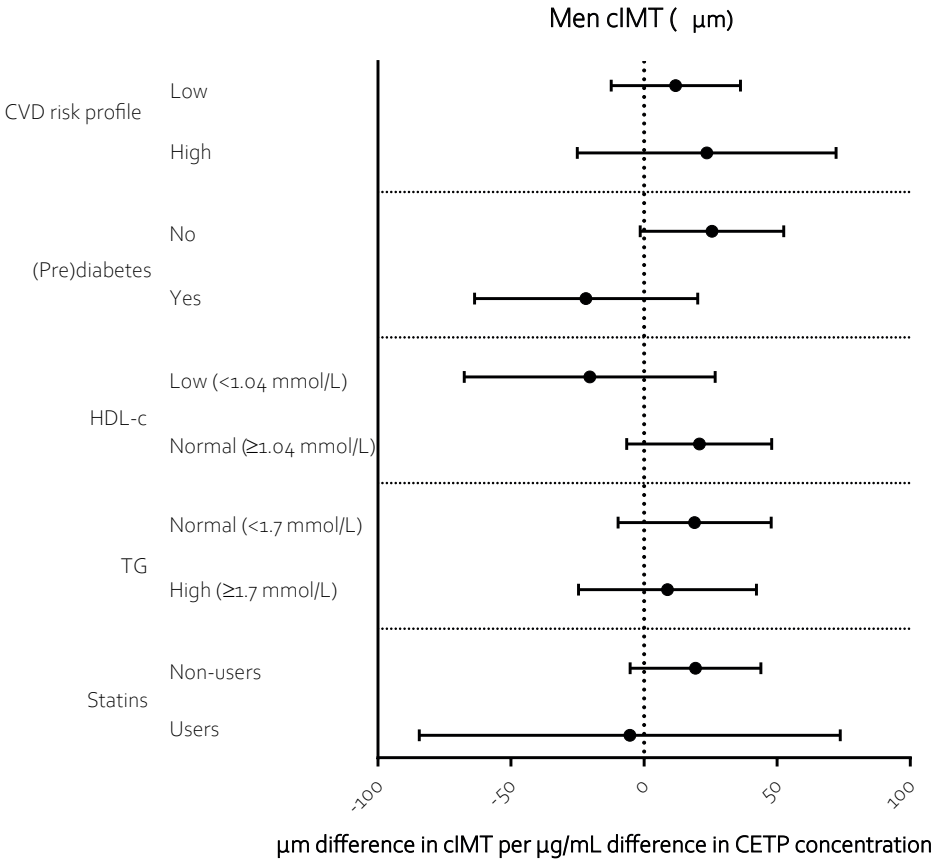


Figure 2 – Associations between (genetically determined) serum CETP concentrations (µg/mL) and intima media thickness (µm) in men participating in the Netherlands Epidemiology of Obesity study, aged 45-65 years (n=2,715). CETP, cholesteryl ester transfer protein; cIMT, carotid intima media thickness; CVD, cardiovascular disease; HDL-c, high density lipoprotein cholesterol; TG, triglycerides.

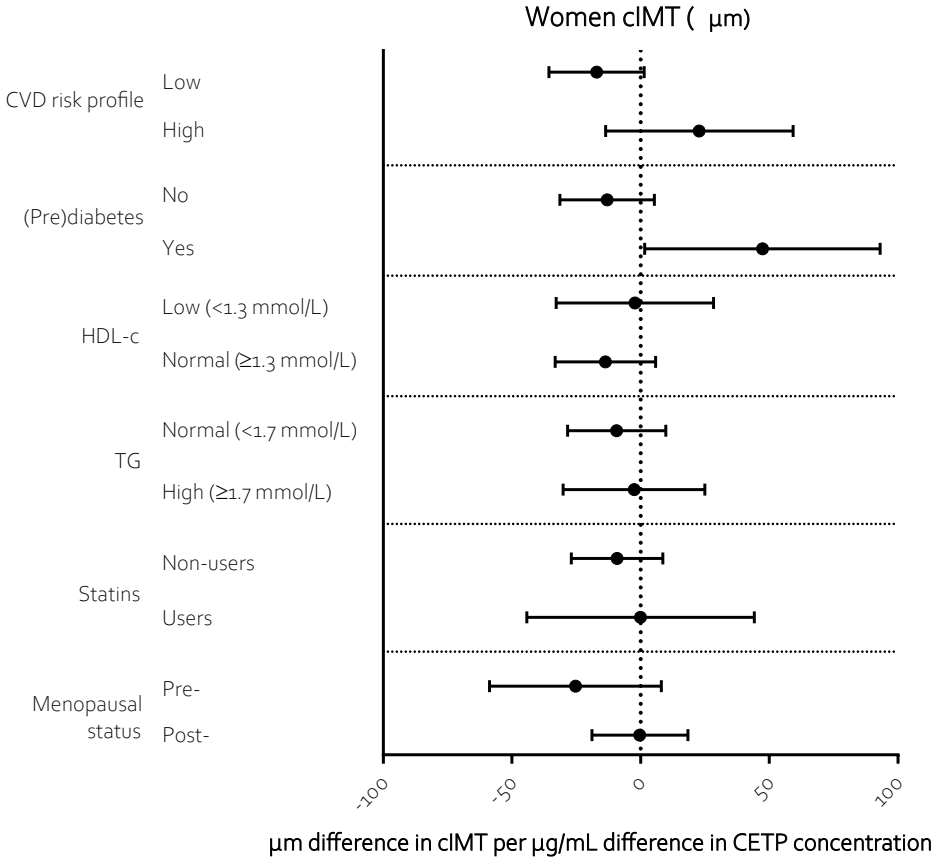


Figure 3 – Associations between (genetically determined) serum CETP concentrations (µg/mL) and intima media thickness (µm) in women participating in the Netherlands Epidemiology of Obesity study, aged 45-65 years (n=2,940). CETP, cholesteryl ester transfer protein; cIMT, carotid intima media thickness; CVD, cardiovascular disease; HDL-c, high density lipoprotein cholesterol; TG, triglycerides.

Discussion

In 5,655 men and women, we investigated the causal associations between CETP and measures of subclinical atherosclerosis. We showed that there was a marginal observational association between CETP and subclinical atherosclerosis in the study population, while a genetic propensity towards higher CETP concentrations was not associated with cIMT. However, stratified analyses in prespecified subgroups of sex, cardiovascular risk, (pre)diabetes, HDL-c, triglycerides and statin use suggested a marginal association between higher CETP and thicker cIMT in men, and a minimal association in the opposite direction in women, albeit with wide confidence intervals.

The null results in the total study population of this study are consistent with findings in the total population in previous Mendelian randomisation studies and randomised controlled trials, however some Mendelian randomisation studies did not measure CETP concentrations. [49, 181, 186-188] Our findings indicate that if a relation between CETP concentrations and subclinical measures of CVD is present, this may be restricted to men, and to women with a moderate-to-high cardiovascular risk or (pre)diabetes in a middle aged population. Our results in high-cardiovascular risk individuals are consistent with the results of the REVEAL trial, that showed a small decrease in incidence of major coronary events in patients with atherosclerotic vascular disease treated with anacetrapib, compared with placebo. [48] These findings are consistent with the positive relations between CETP and subclinical and clinical CVD that were observed mainly in studies in predominantly men.[20, 50, 176, 179, 189] Our findings in women with (pre)diabetes are supported by a smaller previous study that reported that higher observed CETP concentrations were associated with CVD risk in women with type 2 diabetes, but we observed subgroup effects in men that were not observed by this previous study.[196]

CETP mediates the transfer of cholesteryl ester from HDL towards LDL, which is generally assumed to be detrimental with respect to the development of CVD. [197, 198] The surprising absence of an association between CETP and subclinical atherosclerosis on a population level is consistent with the failure of the recent ILLUMINATE[199], dal-OUTCOMES[46], and ACCELERATE[47] clinical trials of pharmacologic CETP inhibition, due to futility or inferiority. However, the ineffectiveness of drugs that target an increase in HDL-c via CETP in the general population may be explained by opposing effects in subgroups that may be masked in summary effect estimates. Although most clinical trials may likely not be powered to perform stratified analyses, reporting stratum-specific effect estimates may reveal different relations in subgroups.

The observed sex differences may be explained by a difference in the relative contribution of a detrimental lipoprotein profile to atherosclerosis in men and women of the same age. This may be an explanation for the observation that women develop atherosclerosis and CVD later than men.[200] It has been shown that while cholesterol is a slightly stronger risk factor in men, diabetes is a stronger cardiovascular risk factor in women than in men.[201] Therefore, women of the same age may need an accumulation of multiple risk factors that are detrimental to the vascular endothelium to develop atherosclerosis. This may be due to vasoprotective effects of endogenous oestrogens,[202] which is consistent with the marginally negative effects that we observed in premenopausal women, and no relation in postmenopausal women. Consequently, the underlying mechanisms of differing relations between CETP and cIMT may reach further than changes in lipid metabolism that are associated with endogenous oestrogens.

Strengths of this study are the large study population with measurements of CETP, cIMT and genetic information to perform a Mendelian randomisation study in the general population. Moreover, extensive phenotypic data was available. Therefore, we were able to perform several subgroup analyses based on sex, menopausal status, medication use and cardiovascular risk factors. The present study also has some limitations that need to be considered. First, the use of cIMT as a measure of subclinical atherosclerosis may limit interpretation of the results in terms of cardiovascular risk. However, previous research has indicated that cIMT is strongly associated with the incidence of future cardiovascular disease in the general population.[203] Second, performing several subgroup analyses increases the risk of chance findings. Further research is needed to replicate the observed relations. Third, this study was performed in a white middle-aged population. Genetic variants or the effects of CETP on atherosclerosis may be specific for this kind of population, and the results therefore need to be confirmed in other ethnicities.

The causal role of CETP is subject to controversy and at the moment of conceptualizing this study, three of four experimental studies of CETP inhibition had been halted due to a lack of protective effect. [204-206] If additional research confirms the potential subgroup effects of CETP, this may indicate that personalisation of pharmacotherapy may be a sensible strategy in the primary and secondary prevention of (recurrent) cardiovascular disease. Further research may elucidate the efficacy of intervening on CETP in subgroups of clinical trials that have been performed. We conclude that despite the absence of a relation between CETP and atherosclerosis in a population of men and women, this relation may be present in men with normal glucose, HDL-c and triglyceride concentrations, and women with a high cardiovascular risk profile or impaired

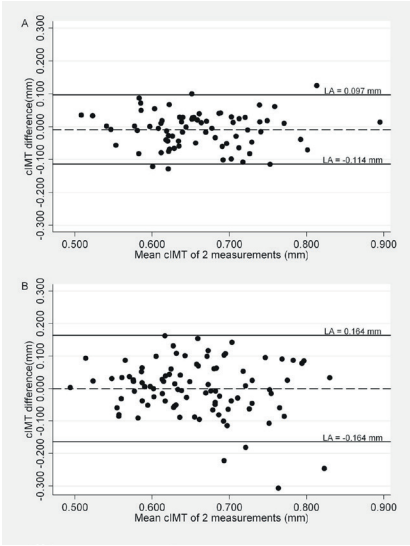
fasting glucose. Further research may give insight in potential sex differences in the aetiology of atherosclerosis.

Appendix

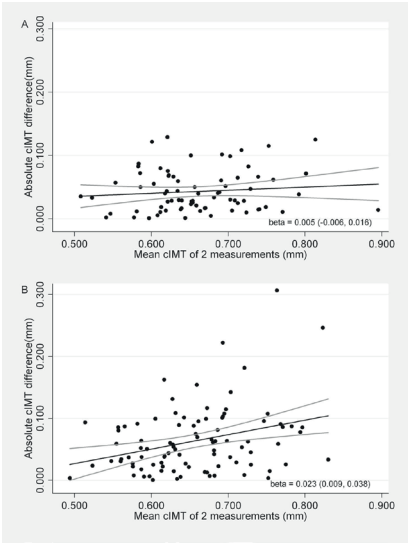
Within the NEO study, cIMT was determined in all participants. Precision of cIMT measurements is crucial to prevent misclassification, decreased study power and diluted effect estimates. We examined the reproducibility of the cIMT measurements in the common carotid artery. We performed paired scans of the cIMT in a random sample of participants at the baseline of the study. Of 169 participants with repeated measurements, 76 participants were scanned twice by the same sonographerm, and 93 participants were scanned twice by two different sonographers. Sonographers were blinded for the scan of the other sonographer. All sonographers were right-handed research nurses who recently had been trained to measure cIMT by an experienced sonographer according to standardized procedures.

To study agreement within and between sonographers we constructed Bland-Altman plots and calculated 95% limits of agreement (LA), intraclass correlation coefficients (ICCs) and coefficients of variation (CVs) for intra-observer and inter-observer measurements separately. For the Bland-Altman plots we calculated the mean cIMT of 2 paired measurements and the difference between 2 paired cIMT measurements by subtracting the second measurement from the first measurement. The ICC explains how much of the total variance between measurements is caused by true inter-subject variability. An ICC of one represents perfect agreement and ICC of zero implies no agreement at all. The CV expresses the standard error between paired measurements as a percentage of the sample mean, where a CV of 0% equals perfect agreement and a CV of 100% signifies no agreement at all. We calculated the ICC and CV for the intra-observer measurements, as well as for the inter-observer measurements.

The mean difference for the intra-observer measurement was -0.009 mm (SD 0.054, 95% LA -0.114 mm to 0.097 mm, Supplementary figure 1, Panel A) and the mean absolute difference was 0.043 mm (SD 0.033). Sixty-nine (91%) intra-observer measurements had an absolute cIMT difference between paired measurements of less than 0.100 mm. The intra-observer ICC was 0.74 (95% CI 0.62 to 0.83) and the CV was 5.8%. The inter-observer measurements had a mean difference of 0.000 mm (SD 0.084, 95% LA -0.164 mm to 0.164 mm, Supplementary figure 1, Panel B) and the mean absolute difference was 0.064 mm (SD 0.054). Seventy-seven (83%) inter-observer measurements had an absolute cIMT difference between paired measurements of less than 0.100 mm. The inter-observer ICC was 0.49 (95% CI 0.33 to 0.63) and the CV was 9.0%.



Supplementary figure 1 – Bland-Altman plots representing the difference between repeated cIMT measurements by the absolute mean cIMT of the repeated measurement. Solid lines represent 95% limits of agreement (LA). Panel A presents the intra-observer variation, panel B presents the inter-observer variation.



Supplementary figure 2 – Plots representing the absolute difference in repeated cIMT measurements by the mean of the repeated measurements. Grey solid lines represent the 95% confidence intervals of the regression line. Panel A represents the intra-observer variation, panel B represents the inter-observer variation.