Genetic and biochemical risk factors in coronary artery disease
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CETP, HDL-c, and cardiovascular risk: Will CETP inhibition translate into cardiovascular risk reduction?

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Introduction
Prospective epidemiological studies have shown unequivocally that low levels of high-density lipoprotein cholesterol (HDL-c) constitute a powerful risk factor for coronary artery disease (CAD). The assumption that raising HDL-c levels will inevitably translate into cardiovascular risk reduction has made HDL-c raising therapy a Holy Grail in pharmaceutical research to date. Over the past two decades, small success in raising of HDL-c levels has been achieved, but mostly as a side effect of drugs developed to modify other lipids. For instance, the low-density lipoprotein cholesterol (LDL-c) lowering effects of statins are accompanied by a modest increase of HDL-c levels. Also, triglyceride-lowering therapy with gemfibrozil raises HDL-c levels to a limited extent. Recently, extensive efforts have been invested in the development of inhibitors of cholesteryl ester transfer protein (CETP), a protein with a pivotal role in cholesterol transport between lipoproteins in the circulation. CETP is a glycoprotein that is physically associated with HDL particles. CETP redistributes cholesteryl esters (CE) and triglycerides between plasma lipoproteins in a process that results in equilibration of these lipids between lipoprotein fractions. It does so by facilitating the transfer of CE from HDL to apolipoprotein B (apoB)-containing particles (i.e., very low density lipoprotein (VLDL) and LDL). In order to keep this transfer energy-neutral, it is reciprocated by transfer of triglycerides from VLDL particles to LDL and HDL lipoproteins. Thus, CETP enables HDL-derived CE to be transferred to apoB-containing lipoproteins and to be taken up by the liver through receptor-mediated uptake, thereby facilitating the anti-atherogenic reverse cholesterol transport (RCT) pathway, i.e. the flux of cholesterol away from peripheral tissues. However, this action results in the transfer of CE from the anti-atherogenic HDL lipoproteins to atherogenic VLDL and LDL fractions, which can be regarded as atherogenic. It remains unclear which of these two properties of CETP predominates in humans, and thus whether inhibition of CETP activity may have anti-atherogenic effects. Substantial evidence from animal studies suggests that the relative importance of the pro- and anti-atherogenic effects of CETP activity may be determined by triglyceride levels, the substrate for CETP activity. The physiological functions of CETP have been described extensively. This review will focus on epidemiological data about the relationship between CETP, HDL-c and CAD risk, and on the results of the first trials of CETP inhibition in humans.

CETP and cardiovascular risk
The inverse association between CETP activity and HDL-c levels is well established, as is the inverse relationship between HDL-c levels and CAD risk. In the 1980s and 1990s, several articles reported that people with hyperalphalipoproteinemia were found to carry mutations in the CETP gene. Pharmaceutical companies have jumped to the development
pharmacological inhibitors of CETP, under the assumption that these high HDL-c levels would translate into decreased CAD risk. Until very recently, however, this hypothesis has not been supported by convincing data. The direct relationship between CETP and CAD risk has been investigated using several different approaches. First, several attempts have been made to assess the risk of CAD among people with CETP gene mutations as the underlying cause of these elevated HDL-c levels.13-17 However, the relationship between reduced CETP function and the susceptibility to atherosclerosis has proven complex and confusing as both longevity and increased CAD risk have been reported13,17-19 Hirano et al. have shown that in people with hyperalphalipoproteinemia, reduced CETP function in conjunction with reduced hepatic lipase activity is associated with an increased risk for CAD.20 Because homozygous CETP-deficient subjects (most of whom live in Japan) are very rare, studies investigating this relationship have focused on people with heterozygous CETP deficiency. An analysis from the Honolulu Heart Study performed among Japanese Americans living in Hawaii suggested that people with heterozygous CETP deficiency had a lower risk of CAD but only if they had HDL-c levels above 60 mg/dl.21 A more recent report with data available after extended follow-up suggested that the risk of CAD may be lower in CETP-deficient heterozygotes, but the trend was not statistically significant.22 Thus, whether homozygous or heterozygous CETP deficiency translates into a reduction in CAD risk remains unclear. Because people with partial or complete CETP deficiency are rare, these studies tend to be small. Therefore, studies using hard cardiovascular endpoints are usually underpowered to detect an effect of CETP deficiency on CAD risk. Using surrogate markers of atherosclerosis, such as intima media thickness (IMT), may provide a solution for this dilemma. We have recently described a Dutch family with CETP deficiency caused by a novel intronic mutation (In7+1) and observed that carriers had similar IMT levels as family members not carrying the mutation.23 These data suggest that CETP deficiency does not lead to an increased risk of CAD. It should be recognized, however, that these studies do not answer the question as to whether CETP inhibition under dyslipidemic or otherwise atherogenic conditions may exert beneficial effects.

A second approach to investigate this issue is by studying genetic variants in the CETP gene. The CETP gene contains many non-coding variants, of which the TaqIB variant has received by far the most attention.24 We have shown in a meta-analysis that people homozygous for the less common B2 allele have on average 0.43 mg/l lower CETP levels (p < 0.00001) and 24 nmol/l/hr lower CETP activity (p < 0.00001) than people homozygous for the B1 allele.25 Consistently, these people also had 0.12 mmol/l higher HDL-c levels (p < 0.00001). Because this genetic variant is associated with CETP levels and HDL-c levels in the absence of a functional effect on the amino-acid sequence or promoter region, it has been suspected to constitute a marker for another functional variant. The -629C>A variant is a good candidate to explain the association between the TaqIB genotype
and HDL-c levels because strong linkage disequilibrium exists between these variants and because the -629C>A variant has been shown to modify CETP promoter activity. Numerous studies have investigated the association between this variant and CAD risk, with inconsistent results. We have recently performed a meta-analysis of individual patient data derived from the largest studies investigating this association. Based on data from 13,667 individuals, and using a multivariate linear regression model that adjusted for all traditional cardiovascular risk factors, we observed that people homozygous for the B2 allele had 0.11 mmol/l (0.10-0.12, p < 0.0001) higher HDL-c levels than B1B1 individuals. Consistently, B2B2 individuals had an odds ratio for CAD of 0.78 (0.66-0.93) compared to B1B1 individuals (p for linearity = 0.008). This association disappeared upon additional adjustment for HDL-c levels. Thus, the association between this genetic variant in the CETP gene and HDL-c levels, does indeed translate into a reduced risk of CAD.

A third approach to assess this issue is by studying the relationship between CETP plasma levels and CAD risk. Although several studies have assessed the relationship with surrogate markers of CAD, large-scale prospective studies investigating the association between plasma CETP levels and cardiovascular endpoints were, until very recently, lacking. Patients with high CETP levels had faster progression of CAD as quantified by angiographic parameters, and accelerated carotid intima-medial thickening. Another small study among Japanese patients undergoing angiography found no association between CETP levels and the extent of coronary atherosclerosis. We have recently performed the first large prospective study investigating the relationship between CETP plasma levels and the risk of future CAD among apparently healthy men and women. We observed that the risk of CAD increased with increasing CETP quintiles (P for linearity=0.02), such that subjects in the highest quintile had an adjusted odds ratio of 1.43 (95% CI 1.03 to 1.99, P=0.03) versus those in the lowest. Among individuals with triglyceride levels below the median of this cohort (1.7 mmol/L), no relationship between CETP levels and CAD risk was observed (P for linearity=0.5), but this relationship was present among those with high triglyceride levels (P for linearity=0.02), such that those in the highest CETP quintile had an OR of 1.87 (95% CI 1.06 to 3.30, P=0.02). This report provides the first epidemiological evidence for a direct relationship between CETP levels and CAD risk.

**Cholesteryl ester transfer protein and triglyceridemia**

The relationship between CETP activity and the lipoprotein and cholesterol metabolism is complex because they are mutually dependent; CETP activity affects the distribution of cholesterol and triglycerides across lipoproteins but is itself, in turn, directed by the composition of various lipoproteins. In several human dyslipidemias associated with accelerated atherosclerosis, CETP levels and/or the rate of net transfer of CE from HDL to apoB-containing lipoproteins are increased as recently summarized in
a review. Briefly, CETP levels were increased in people with hypercholesterolemia, dysbetalipoproteinemia, and severe chylomicronemia. This might imply that CETP is deleterious, but it can also be argued that high CETP levels are the result rather than the cause of dyslipidemia. Lifestyle factors further complicate this issue: it has been described that alcohol abuse and physical exercise, typically associated with increased HDL-c, are associated with diminished CETP concentration. Similarly, smoking (associated with low HDL-c) is associated with high CETP activity. A cross-sectional study showed an inverse relation between CETP and HDL-c among hypertriglyceridemic but not normotriglyceridemic men. These studies clearly indicate that plasma CETP levels are affected by a variety of metabolic conditions and lifestyle factors that are in themselves associated with changes in CAD risk.

To complicate matters even further, the ultimate outcome in terms of cardiovascular risk may depend not only on CETP activity itself, but also on the presence of its substrate: triglycerides. For instance, under normotriglyceridemic circumstances, CETP activity results in the preferential transfer of CE from HDL to LDL particles. It is anticipated that under these circumstances CETP activity mainly affects HDL-c and LDL-c levels. However, under hypertriglyceridemic conditions caused by an enhanced production of VLDL particles such as in type 2 diabetes, the strong disequilibrium of triglycerides across lipoproteins results in a shift of the preferential CETP-mediated transfer of HDL-derived CE from LDL to VLDL. In addition, the large pool of triglycerides in VLDL particles results in a CETP-mediated transfer of triglycerides from VLDL to LDL and a reciprocal shuttle of CE from LDL to VLDL. The resulting cholesterol-depleted LDL particles have an enriched triglyceride-content which can undergo hydrolysis leading to a further reduction in LDL size. Thus, CETP facilitates the formation of small dense LDL particles, but this reaction requires the driving force of a substantial pool of triglycerides in VLDL particles. Some investigators have suggested that the formation of small dense LDL requires a threshold triglyceride concentration of approximately 1.5 mmol/L.

The complexity of the role of CETP activity in cholesterol metabolism is underlined by the inconsistency of observational studies on the relationship between CETP levels and cardiovascular risk. For instance, CETP levels or activity have been described to be increased, unchanged, and decreased in type 2 diabetes, which tends to be associated with hypertriglyceridemia. In the DAIS study, which tested the efficacy of fenofibrate therapy among patients with type 2 diabetes, baseline CETP concentration was associated with an unfavourable lipid profile characterized by increased triglycerides, VLDL triglycerides and smaller LDL size. After 3 years of placebo treatment, however, CETP concentration was not associated with increased progression of coronary atherosclerosis as quantified by surrogate angiographic markers. In the fenofibrate group, by contrast, baseline CETP concentration was positively
associated with the progression of coronary atherosclerosis. In the absence of a direct effect of fenofibrate on CETP concentration, it remains to be established how CETP and fenofibrate treatment interact to influence the progression of coronary atherosclerosis. The results point towards a better cholesterol-lowering capacity of fenofibrate in patients with low CETP concentration, but this needs further confirmation. Above all, these findings underline our incomplete understanding of the relationships among CETP levels, triglycerides, and atherosclerosis. Among men with established CAD, high CETP concentration were associated with faster progression of coronary atherosclerosis and with a better response to pravastatin therapy. Among patients with familial hypercholesterolemia, CETP levels were also positively associated with a more atherogenic lipid profile and increased progression of atherosclerosis. However, CETP levels were now associated with worse response to pravastatin treatment. These inconsistent observations may derive from chance or limited statistical power, but they may also reflect a true difference of the role of CETP in different patient categories.

**Cholesteryl ester transfer protein inhibition in humans**

To date, the strategies used to inhibit CETP activity have been by autoantibody-mediated inhibition and by small molecule inhibitors (JTT-705 and torcetrapib). First of all, a vaccine has been shown to inhibit CETP by initiating the production of auto-antibodies against the protein. After promising results in cholesterol-fed rabbits, the safety and immunogenicity was recently tested in a cohort of 48 healthy subjects with low levels of HDL-c (<1.55 mmol/l). The vaccine was well tolerated and induced the generation of auto-antibodies in more than half the patients after a second injection. However, no significant inhibition of CETP activity or increase in HDL-c level was noted in the immunized subjects. It has been suggested that the dosing schedule was suboptimal for reaching effective antibody titers. Phase II trials have been initiated to further explore this approach.

JTT-705 inhibits CETP activity by forming a disulphide bond with the protein. JTT-705 was initially tested in phase I studies with doses ranging from 100 mg to 1800 mg. These studies showed that the drug was well tolerated and did not result in significant toxicity in healthy Caucasian men. A two-period crossover bioavailability study revealed that the drug induced a more pronounced CETP inhibition in the postprandial phase compared with the fasted state. In a subsequent randomized, double-blind placebo-controlled phase II trial, JTT-705 was tested in 198 healthy subjects with mild hyperlipidemia for 4 weeks. Daily administration of JTT-705 900 mg resulted in an increase of HDL-c levels (33.9%, p< 0.001) and a decrease of LDL-c levels (7.4%, P=0.012) compared to placebo. The drug proved safe and was well tolerated and no adverse events were reported. Recently, a second phase II, double-blind, placebo-controlled trial tested the efficacy of JTT-705 on top of pravastatin treatment. Patients were treated with
pravastatin 40 mg in combination with placebo, JTT-705 300mg or 600 mg. After 4 weeks of combined treatment, JTT-705 600 mg induced a 30% decrease in CETP activity from baseline (p < 0.001), which was accompanied by a 28% increase in HDL-c level (p < 0.001). In addition, a 5.5% decrease in LDL-c was noted (P = 0.03). Total cholesterol, triglycerides, apolipoprotein B and apolipoprotein E levels were similar across the treatment groups. The combination therapy of JTT-705 with pravastatin was well tolerated and did not induce significant adverse effects.

The second investigational CETP inhibitor, torcetrapib, has been evaluated in two small, non-blinded, placebo-controlled studies. A phase I study tested torcetrapib 10 mg, 30 mg, 60 mg, 120 mg once daily, and 120 mg twice daily in 40 healthy normolipidemic subjects. The increases in HDL-c levels ranged from 16% (10 mg) to a striking 91% (120 mg twice daily) from baseline. This latter result was accompanied by a 42% decrease in LDL-c.59

In the second study, 19 patients with low HDL-c levels (<1.0 mmol/l) were treated with torcetrapib 120 mg alone or in combination with atorvastatin 20 mg (n=9).60 Torcetrapib 120 mg increased plasma HDL-c levels by 46% and 61% in the torcetrapib alone and combination groups, respectively. In six subjects who received torcetrapib 120 mg twice daily for an additional 4 weeks, HDL-c levels were raised by 106%. Torcetrapib reduced LDL-c levels in the atorvastatin group by an additional 17%. Interestingly, torcetrapib 120 mg once and twice daily increased the concentrations of large LDL particles by 257% and 294%, respectively, compared to the placebo. These changes were accompanied by a concomitant 73% and 93% decrease in small LDL particles, respectively. Dosages ranging from 10 mg to 240 mg daily were well tolerated, and there were no serious adverse events and no withdrawals due to adverse events.

The comparison of JTT-705 and torcetrapib monotherapy reveals an intriguing difference. Among people with mild hyperlipidemia, JTT-705 900 mg daily reduced CETP activity by 37%, which resulted in a 34% increase of HDL-c levels.58 However, in individuals with low baseline HDL-c (<1.0 mmol/l), 120 mg torcetrapib daily induced a 46% increase of HDL-c with only 28% CETP inhibition.59 Thus, the percentage CETP inhibition needed to raise HDL-c by 1% was 0.61% in the torcetrapib-treated individuals but 1.09% in the JTT-705-treated individuals. Perhaps these data teach us that CETP inhibition is more effective for increasing HDL-c in people with low HDL-c levels than in subjects with relatively normal HDL-c levels, an issue that needs further investigation. Torcetrapib 120 mg proved safe and effective when used in combination with atorvastatin 20 mg in 9 individuals with low HDL-c. This is important, considering that in clinical practice HDL-c raising therapy is likely to be used in combination with evidence-based LDL-c-lowering medication. This study again underscored the potency of this drug to reduce LDL-c levels: in six individuals who used 120 mg torcetrapib twice daily LDL-c was reduced by 17%. A similar reduction was achieved in nine individuals who received the combination treatment. In addition to their HDL-c increasing potential, JTT-705 and torcetrapib share the absence
of effect on plasma triglyceride levels (moderate at the highest dosages of torcetrapib). It will be interesting to see how these investigational drugs will modulate lipids and lipoproteins in hypertriglyceridemic individuals. Another notable finding is that both inhibitors cause a marked increase in plasma CETP concentration. Clark et al. suggested that this is the result of an inhibitor-induced complex formation between CETP and HDL.\textsuperscript{59} Although this complex formation was not assumed to affect HDL function, the biological properties of the markedly changed HDL pool, complexed with CETP inhibitors or not, have not been addressed to date. In addition, there is no mention of the effects of CETP inhibitors on the excretion of cholesterol and bile acids as a final step in proposed RCT pathway.

**Unresolved issues**

Despite fast progress in the field of pharmacological inhibition of CETP, many issues remain unresolved about its physiological function in cholesterol metabolism, its pathophysiological role in atherogenesis, and the potential consequences of CETP inhibition. For instance, HDL particles may have atheroprotective properties because they facilitate the RCT pathway of cholesterol from the vessel wall back to the liver. CETP inhibition raises HDL levels but whether these altered HDL particles remain effective acceptors of free cholesterol from lipid-laden macrophages, the crucial first step in the RCT pathway, has not been established. This is of interest because in CETP-deficient Japanese subjects CE-enriched HDL cannot protect macrophages from cholesterol accumulation.\textsuperscript{61} On the other hand, HDL from JTT-705-administered rabbits was able to reduce CE concentration in J774 macrophages as efficiently as that from control rabbits.\textsuperscript{62} Very recently, it was shown that two ATP-binding cassette transporters (ABCG1 and ABCG4) can mediate cholesterol efflux to both smaller (HDL-3) and larger (HDL-2) particles in vitro.\textsuperscript{63} This suggests that large HDL particles may still be able to accept cholesterol efflux from lipid-laden macrophages. Second, it remains to be determined whether CETP inhibition results merely in a redistribution of CE and triglycerides among lipoproteins, or whether it in fact results in flux of CE through the RCT pathway, ultimately resulting in increased excretion of cholesterol into the bile. Unfortunately, very little is known about the functionality of the scavenger receptor class B type 1 (SR-B1) in humans, which in mice mediates the selective uptake of HDL lipids by the liver.\textsuperscript{64} Recently, it was observed in vitro that large HDL particles bind better to SR-B1 than smaller particles.\textsuperscript{65} If this is also the case in vivo in the human liver, it can be hypothesized that CETP inhibition, which results in larger HDL particles, may lead to a normal or even an increased uptake of CE from HDL particles. Furthermore, little is known about the hepatic mechanisms that facilitate cholesterol excretion into the bile. Whether an increased amount of cholesterol recruited from the periphery results ultimately in an increased amount of cholesterol excreted into the bile, requires further research. Finally, the atheroprotective role of HDL-c extends beyond mediating RCT.
It will therefore be important to assess whether CETP inhibition affects the anti-inflammatory and anti-oxidative properties of HDL particles.

**Conclusion**

We conclude that the current CETP inhibitors have cleared the first hurdle in that they have proven safe and efficacious in increasing HDL-c levels. In addition, both JTT-705 and torcetrapib have been shown to be effective and safe when used in combination with evidence-based statin therapy to lower LDL-c. Whether CETP inhibition results in a reduced risk of CAD is a hypothesis that needs to be tested in large randomized controlled trials. Although the information from observational studies is somewhat confusing, most evidence suggests that under physiological circumstances, lower CETP levels may have a beneficial effect on cardiovascular risk. However, substantial evidence suggests that these effects may be substantially different in various metabolic contexts. Therefore, further research into the physiological and pathophysiological roles of CETP in various dyslipidemias remains essential to gain insight into which patient categories may benefit from CETP inhibition.

In summary, given the complexity of the role of CETP in cholesterol metabolism, we cannot rule out that CETP inhibition may have different effects in different patient categories. However, despite the fact that CETP inhibitors have only been tested in approximately 300 individuals, the current results indicate that CETP inhibition has the potential to be of great importance in the future prevention of cardiovascular disease.
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