Monogenic causes of elevated HDL cholesterol and implications for development of new therapeutics

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Abstract

Identification of the CETP, LIPG (encoding endothelial lipase) and APOC3 genes, and analysis of rare genetic variants in them, have allowed researchers to increase understanding of HDL metabolism significantly. However, development of cardiovascular risk-reducing therapeutics targeting the proteins encoded by these genes has been less straightforward. The failure of two CETP inhibitors is complex but illustrates a possible over-reliance on HDL cholesterol as a marker of therapeutic efficacy. The case of endothelial lipase exemplifies the importance of utilizing population-wide genetic studies of rare variants in potential therapeutic targets to gain information on cardiovascular disease end points. Similar population-wide studies of cardiovascular end points make apoC-III a potentially attractive target for lipid-related drug discovery. These three cases illustrate the positives and negatives of single-gene studies relating to HDL-related cardiovascular drug discovery; such studies should focus not only on HDL cholesterol and other components of the lipid profile, but also on the effect genetic variants have on cardiovascular end points.

Keywords

anacetrapib; apoC-III; CETP; cholesterol; dalcetrapib; endothelial lipase; evacetrapib; genetics; HDL; ISI-APOCIII₉X; torcetrapib

Over the past several decades, the widespread use of statins to lower LDL cholesterol (LDL-C) levels has contributed to a substantial decrease in the incidence of coronary heart disease (CHD) [1]. Despite this, considerable residual CHD risk remains, even in patients with optimal LDL-C [2,3]. Consequently, substantial research has been directed toward identifying additional lipid metabolism targets that have the potential to further decrease cardiovascular risk. Studies of human genetics have featured prominently in these
endeavors, revealing previously unknown biology that has led, in turn, to the development of new therapeutic targets. For example, linkage analysis in families with autosomal dominant hypercholesterolemia of unknown etiology led to the identification of gain-of-function mutations in PCSK9 [4]; subsequent targeted sequencing of PCSK9 in individuals with extremely low LDL-C identified loss-of-function mutations [5] that were found to be protective against CHD [6]. Based on the strength of this human genetic data, PCSK9 is a major target for pharmacologic inhibition as a means of lowering LDL-C, with promising early-phase results [7].

In addition to LDL-C, HDL cholesterol (HDL-C) has been an attractive focus of research since it was identified as having an inverse relationship with CHD risk in epidemiological studies [8]. However, despite a great deal of research and significant financial investment into pharmaceutical development, the feasibility of preventing cardiovascular disease through strategies that increase HDL-C remains unclear [9]. Despite the fact that the high HDL-C phenotype is often of polygenic origin [10], research into single-gene conditions that increase HDL-C could lead to new therapeutic targets. This review examines three known monogenic causes of elevated HDL-C: loss-of-function mutations in CETP (encoded by CETP), endothelial lipase (EL; encoded by LIPG) and apoC-III (encoded by APOC3). CETP transfers cholesteryl esters from mature HDL particles to atherogenic apoB-containing lipoproteins, such as LDL and VLDL, in exchange for triglycerides (TGs) [11]. EL hydrolyzes HDL phospholipids, thus destabilizing the HDL particle, leading to increased catabolism [12]. ApoC-III inhibits lipoprotein lipase (LPL), leading to decreased hydrolysis of TG-laden cholesterol particles and a reciprocal decrease in HDL-C. Examination of these three conditions elucidates the critical role studies of genetic variants in single genes can play in improving our understanding of HDL metabolism. They also emphasize the essential nature of such genetic studies in identifying drug targets; indeed, the recognition that loss-of-function mutations in these three genes lead to increased HDL-C levels placed all three genes on a short-list of potential therapeutic targets for raising HDL-C. However, these examples also emphasize the fact that translational research on newly identified HDL-related genes must focus not only on HDL-C concentration, but also on HDL function, measures of atherosclerosis and cardiovascular disease end points.

**CETP**

**CETP deficiency, observational epidemiology & genetic variation**

CETP was first purified and characterized in 1987 [13]. Three years later, two Japanese siblings with extremely high HDL-C levels and increased HDL size were found to lack CETP due to loss-of-function mutations [14]. A larger investigation identified the same mutations causing CETP deficiency in four out of 11 families with high HDL-C levels [15]. Subjects homozygous for the mutation had markedly increased HDL-C and apoA-I levels, together with decreased LDL-C and apoB levels, while heterozygotes showed modestly elevated HDL-C levels. These studies solidified CETP as a protein of interest for lipoprotein metabolism and identified it as a therapeutic target for inhibition as a strategy to raise HDL-C.
Many subsequent investigations of CETP's function focused on its association with both pro- and anti-atherogenic cardiovascular markers. CETP activity has been shown to correlate directly with LDL-C and non-HDL-C, and inversely with HDL-C, in patients with hyper-cholesterolemia and combined hyperlipidemia [16], and with serum LDL-C (but not HDL-C) in healthy Japanese patients [17]. Multiple studies have also identified a correlation between increased CETP concentrations and various dyslipidemic states, such as dysbetalipoproteinemia, severe chylomicronemia, nephrotic syndrome and familial hypercholesterolemia [18–20], as has been reviewed elsewhere [11].

Evaluation of CETP's association with atherosclerosis and CHD is less straightforward. It has been posited that CETP may in fact be antiatherogenic and that dyslipidemia causes elevated CETP, rather than the converse [21]; some preclinical data substantiates the hypothesis that CETP plays a role in reverse cholesterol transport [22,23]. There have been several observational studies of the association of plasma CETP mass and/or activity with CHD. A nested case–control study of 111 male CHD cases and 116 male CHD-free controls found that while plasma mass CETP levels were not significantly different between the two groups overall, a subgroup analysis of subjects with low TGs (below the population mean) found that CETP levels were lower in cases than in controls [24]. A similar case–control study of 114 CHD cases and 105 controls drawn from the same cohort again showed no difference in CETP levels between the two groups, but found that CETP activity was significantly higher in cases than in controls [25]. A prospective study of CETP activity in 2679 subjects found that low levels of activity were associated with increased CHD risk in men, but not in women [26]. Finally, a prospective study of 3256 patients undergoing coronary angiography found that patients in the lowest quartile of plasma CETP mass level had an increased mortality risk when compared with patients in the highest quartile [27].

Studies of the association of genetic variation in CETP with CHD may be more likely to reveal potential causal associations. In early studies, sequencing of 201 Japanese patients with HDL-C ≥100 mg/dl found that 67% carried deleterious mutations in CETP [28]. However, ten of the 12 subjects with atherosclerotic cardiovascular disease were heterozygous for CETP mutations, implying that while CETP deficiency may lead to high HDL-C, it may not be cardioprotective. A later study of 104,505 Japanese subjects by the same group found that those with genetic CETP deficiency due to a splice site mutation and markedly elevated HDL-C did not live longer than those without the mutation, and that the frequency of the mutation was higher in patients with coronary artery disease than in healthy controls [29]. In a smaller population of 3469 Japanese–American men, heterozygotes for CETP deficiency had modestly elevated HDL-C overall, but those who had HDL-C between 41–60 mg/dl had an increased prevalence of CHD [30]. However, a follow-up study showed a lack of a significant association of CETP mutations with incident CHD [31]. The I405V polymorphism in CETP has been associated with low CETP activity and increased HDL-C in a study of 576 men, but also with possibly increased risk of CHD in those with hypertriglyceridemia [32], greater CHD risk in Caucasian women [33] and increased carotid intima–media thickness (CIMT) in men [34].

Despite this, evidence drawn from much larger samples focused on common variants at the CETP locus suggests that genetic reduction in CETP activity not only increases HDL-C and...
decreases LDL-C, but is also associated with a modest reduction in CHD risk. A meta-analysis incorporating 196,367 subjects found that the I405V mutation, a −629C>A polymorphism, and the intronic CETP variant Taq1B were all associated with lower CETP mass and activity, increased HDL-C, increased apoA-I, decreased LDL-C, and a small reduction in CHD risk [35]. However, a pooled analysis of 10,526 undifferentiated subjects and 10,947 subjects at high CHD risk found that carriage of the Taq1B variant was associated with increased CHD risk in the former group, but with decreased risk in the latter group [36]. Another meta-analysis of 12,482 myocardial infarction (MI) cases and 41,331 controls found that a 2490C>A polymorphism at the CETP locus was associated with increased HDL-C, reduced LDL-C and reduced risk of MI [37]. While one prospective study of 6780 subjects found that neither Taq1B nor -629C>A status affected the direct relationship between the apoB/ apoA-I and total cholesterol:HDL-C ratios, and CHD risk [38], another prospective cohort study of 18,245 healthy women found decreased MI risk and increased HDL-C in carriers of the Taq1B, 852 + 783T>C, and 179 + 3052A>G polymorphisms in or near CETP [39]. A third prospective cohort study of 10,261 individuals found that the Taq1B and −629C>A polymorphisms were associated with lower CETP activity, increased HDL-C, decreased LDL-C and a significantly lower risk of ischemic cardio- and cerebro-vascular disease [40].

From the evidence discussed above, the observational epidemiology regarding the association of CETP mass and activity with CHD events is internally inconsistent and conflicts with the genetic data. A potential explanation is that the association of decreased CETP mass with increased CHD events may be confounded – a hypothesis borne out by Mendelian randomization analyses – suggesting that genetic variants at the CETP locus that reduce CETP mass and/or activity appear to modestly reduce cardiovascular risk [35].

**CETP inhibition**

Given CETP deficiency's association with increased HDL-C, pharmacologic inhibition of CETP has been of considerable interest for some time. However, the question of whether such inhibition leads to improved cardiovascular outcomes has not been conclusively answered. Although several preclinical studies detailing the use of antisense oligodeoxynucleotides [41] and a vaccine against CETP [42,43] have been published, pharmacological attention has focused primarily on small-molecule CETP inhibitors.

The first widely studied inhibitor, torcetrapib, was shown to reduce aortic atherosclerosis in rabbits fed an atherogenic diet for 16 weeks [44]. At the study's end, aortic atherosclerosis and aortic cholesterol content were 60% lower in 24 torcetrapib-treated animals than in 23 controls. Early clinical studies focused on torcetrapib's effects on lipoprotein profiles. For example, a Phase I dose-finding study of 40 healthy individuals noted elevations in HDL-C, apoA-I, apoE and TGs along with reduced non-HDL-C, apoB and cholesteryl ester content [45]. Another study in low HDL-C subjects found that torcetrapib administration, both with and without accompanying statin therapy, increased HDL-C levels and particle size significantly, while decreasing LDL-C [46], as did larger randomized controlled trials of low HDL-C subjects [47,48]. However, Phase III evaluations of torcetrapib's effects, not only on lipid levels, but also on atherosclerosis, did not show benefit and, in fact, demonstrated
harm. The ILLUSTRATE trial of 1188 patients with pre-existing coronary artery disease on statin therapy randomized to torcetrapib or placebo found a significant increase in HDL-C and a decrease in LDL-C in the torcetrapib-treated group, but no significant difference in the progression of coronary atherosclerosis, as measured by intravascular ultrasound [49]. The RADIANCE 1 trial of 850 patients with heterozygous familial hypercholesterolemia showed a similarly beneficial effect on torcetrapib-treated subjects’ lipid profiles, but no significant difference in maximum CIMT, and a significant increase in annualized change in mean CIMT compared with the statin-only group [50]. RADIANCE 2, an evaluation of 752 mixed dyslipidemia patients randomized to torcetrapib plus statin or statin alone, also found increased HDL-C and decreased LDL-C in the torcetrapib group, but no significant change in maximum CIMT per year [51]. Torcetrapib development ended following the early cessation of the ILLUMINATE trial of 15,067 patients at high risk for CHD due to an increased risk of cardiovascular events and all-cause mortality in the torcetrapib-treated group [52]. Torcetrapib is now known to have off-target effects, such as stimulation of aldosterone secretion and increased blood pressure, which may have contributed to its adverse effects [53].

The second widely studied CETP inhibitor was the less potent compound dalcetrapib. A study in rabbits found a 40–45% HDL-C increase and a 15–20% LDL-C decrease, as well as a 70% decrease in aortic atheroma lesion area (compared with an 80% decrease in a statin-treated group) [54]. Another rabbit study noted a significant HDL-C increase compared with control; however, no difference in aortic atheroma area was seen, and a correlation analysis found no relationship between atherosclerosis development and either CETP activity or HDL-C level [55]. A Phase II clinical trial of mildly dyslipidemic human subjects showed that increased HDL-C and slightly decreased LDL-C accompanied a dose-dependent decrease in CETP activity [56]. Although the dal-PLAQUE Phase IIb trial found a decrease in MRI-assessed carotid total vessel area in dalcetrapib-treated patients compared with placebo, it showed no difference between groups in other coprimary end points, such as PET:computed tomography most-diseased-segment target:background ratio, MRI-assessed carotid wall area and MRI-assessed normalized wall index [57]. Dalcetrapib development was halted following the early cessation for futility of the Phase III dal-OUTCOMES mortality and morbidity study [58]. In dal-OUTCOMES, 15,871 patients with recent acute coronary syndrome were randomized to dalcetrapib or placebo in a 1:1 ratio and followed for the composite end point of CHD death, non-fatal MI, ischemic stroke, unstable angina or cardiac arrest with resuscitation. The study was halted after interim analysis found that while dalcetrapib increased HDL-C by 31–40% in the treatment group (compared with 4–11% in the placebo group), it did not reduce LDL-C, and, most importantly, did not reduce the risk of recurrent cardiovascular events (hazard ratio for dalcetrapib vs placebo: 1.04; 95% CI: 0.93–1.16; p = 0.52) [58]. Potential explanations for this result include that increased HDL-C through CETP inhibition is not cardio protective, that inhibition of CETP does not promote (and may even impair) reverse cholesterol transport, that the increase in HDL-C was not adequate, that there was no accompanying reduction in LDL-C and that dalcetrapib had off-target effects that offset any protection conferred by the drug.

Despite the lack of promising results for torcetrapib and dalcetrapib, the hypothesis that CETP inhibition is atheroprotective has not been definitively discarded. As noted above,
torcetrapib’s failure has been attributed to off-target effects considered unique to that drug, while dalce-trapib’s lack of efficacy might be attributed to its lack of potency – perhaps it does not raise HDL-C enough to confer clinical benefit. A CETP inhibitor that raises HDL-C to a greater degree without causing harmful off-target effects might yet prove therapeutically useful. Anacetrapib and evacetrapib are CETP inhibitors still in clinical development that might meet these criteria.

Anacetrapib binds CETP significantly more tightly than dalce-trapib in a fashion similar to torcetrapib [59]. Unlike torcetrapib, however, multiple trials have so far found no increased incidence of hypertension or adverse cardiovascular effects in anacetrapib-treated patients. Phase I studies demonstrated increased HDL-C and apoA-I, and decreased LDL-C, without increases in blood pressure [60]. A larger study in patients with primary hypercholesterolemia or mixed dyslipidemia randomized to combination statin and anacetrapib, anacetrapib alone, statin and placebo, or placebo alone found significantly increased HDL-C and decreased LDL-C and lipoprotein(a) (Lp(a)) for all anacetrapib-treated groups compared with placebo, without significant effect on subjects’ blood pressure levels [61]. At the highest doses of anacetrapib, increases in HDL-C of up to 139% were seen, as well as decreases in LDL-C and Lp(a) of up to 40 and 50%, respectively. Similarly, the DEFINE trial of 1623 CHD patients on statin therapy randomized to anacetrapib or placebo showed a significant increase in HDL-C (138%), a significant decrease in LDL-C (40%) and Lp(a) (36%), no changes in blood pressure, aldosterone, or electrolyte levels, and no increased incidence of adverse cardiovascular events in the treatment group when compared with placebo [62]. The 30,000-subject REVEAL trial of anacetrapib in patients with pre-existing cardiovascular disease is scheduled to be completed at the beginning of 2017 [201]. Of note, anacetrapib’s effects on LDL-C – nearly comparable to those of statin monotherapy – and on Lp(a) provide potential mechanisms beyond HDL-C raising by which anacetrapib might be cardio-protective [61]. Dalce-trapib, by contrast, did not have an appreciable effect on LDL-C levels [58].

Evacetrapib was the fourth CETP inhibitor to enter Phase III clinical development. A Phase II study of high LDL-C or low HDL-C patients randomized to evacetrapib at a range of doses, statin alone, evacetrapib/statin combination therapy or placebo found that evacetrapib alone increased HDL-C by 54–129% and decreased LDL-C by 14–36% compared with placebo. Evacetrapib given in combination with a statin increased HDL-C by 79–89% and decreased LDL-C by 11–14% compared with statin monotherapy [63]. Evidence that this improved lipid profile is in fact atheroprotective requires completion of the 11,000-subject ACCELERATE Phase III trial, scheduled for completion in September 2015 [202].

Despite the allocation of considerable resources, the question of whether or not CETP inhibition is a viable strategy to decrease cardiovascular risk has yet to be answered conclusively. As noted above, genetic variants in CETP that reduce its activity not only increase HDL-C but also reduce LDL-C. Similarly, anacetrapib and evacetrapib significantly decrease LDL-C in addition to raising HDL-C. Both drugs also lower Lp(a) through mechanisms that are unclear at present. These effects on LDL-C and Lp(a) may make it difficult to apportion mechanistic responsibility for any cardiovascular benefit from CETP inhibition that may be proven in the future. Should either remaining CETP inhibitor
be shown to decrease cardiovascular risk, it will be unclear whether cardioprotection is due to increased HDL-C, decreased LDL-C, decreased Lp(a) or some combination of all three factors. Cumulatively, these issues highlight the risk inherent in using genetic studies to identify HDL-related therapeutic targets without concurrent examination of atherosclerotic burden and cardiovascular risk, as well as the difficulty of specifically proving whether raising HDL-C has a beneficial effect on cardiovascular risk at all.

**Endothelial lipase**

Analysis of the protracted course of CETP investigation raises an important issue regarding single-gene studies in cardiovascular disease. Such studies have played and will continue to play a critical role in increasing our knowledge of lipid metabolism and identifying targets for drug development. However, cases of major genetic effects in these genes are often too rare to perform rigorous studies of cardiovascular end points. The progression of inquiry into EL provides an alternative model for single-gene investigation in which the decision to pursue drug development is guided by population-level genetic studies of low-frequency variants of large effect. Large-scale genetic research can provide data on the effect single genes have on overall cardiovascular risk prior to the initiation of costly and lengthy clinical trials.

EL (gene name *LIPG*) was first cloned and characterized in 1999 [64,65]; mice overexpressing EL were found to have decreased HDL-C and apoA-I levels [64]. EL destabilizes the HDL particle by hydrolyzing HDL phospholipids. The small, remodeled particle releases lipid-poor apoA-I; both apolipoprotein and HDL particles are subsequently catabolized at an increased rate, resulting in decreased HDL-C and apoA-I [12]. Similar decreases in HDL-C size and particle number, as well as apoA-I levels, have been found in other overexpression studies in mice [66–68]. The inverse relationship between EL activity and HDL-C levels has been reinforced by loss-of-function studies in mice. Inhibition of EL with an antibody significantly increased HDL-C levels in mice [69]. Another study found increased HDL-C in both *Lipg<sup>−/−</sup>* and *Lipg<sup>+/−</sup>* mice due to an increase in the absolute number of HDL particles [68]. Another noted that *Lipg<sup>−/−</sup>* mice have elevated HDL-C, apoA-I and apoE levels, with an increased number of large HDL particles and decreased HDL clearance [70].

Measures of cholesterol efflux and overall reverse cholesterol transport in cell lines and animals are also affected by overexpression and knockout of EL, but not always in the manner one might hypothesize. For example, over-expression of EL in a mouse model led to greatly diminished HDL-C, apoA-I and scavenger receptor-BI-mediated cholesterol bidirectional flux, but also to increased ABCA1-mediated cholesterol efflux, despite the lower amount of apoA-I present; this was attributed to ABCA1’s affinity for small, immature HDL particles of the type formed through EL’s action [66]. In another study, EL overexpression was noted to cause cholesterol efflux from macrophages by promoting apoA-I binding to ABCA1, while EL suppression led to decreased cholesterol efflux [71]. However, a mouse study has found that while EL overexpression led to increased hepatic cholesterol content, it did not increase the excretion of cholesterol into feces [72]. Finally, EL/hepatic lipase (HL) double-knockout mice have been shown to have increased HDL-C
and cholesterol efflux capacity as compared with single-knockout mice, as expected, but did not demonstrate an increased rate of \textit{in vivo} macrophage reverse cholesterol transport [73].

A number of analyses have examined the effect of \textit{LIPG} common variants and rare mutations on lipid levels in human subjects. In a 2002 study, the \textit{EL} gene was sequenced in 20 people with high HDL-C; six coding variants were identified [74]. One variant, T111I, was associated with increased HDL-C in some studies [70,75] but not others [76–79]. The T111I EL variant was shown to have normal lipolytic function \textit{in vitro} [80] and was subsequently found to be in linkage disequilibrium with a promoter single nucleotide polymorphism (SNP) that affects \textit{EL} transcription [81]. Thus, its association with HDL-C, if any, is likely to be due to its strong linkage disequilibrium with a causal SNP that influences transcription.

A second variant, N396S, was subsequently found to be highly significantly associated with increased HDL-C and apoA-I [81]. It was also shown to reduce lipolytic function \textit{in vitro} and \textit{in vivo}. The N396S variant was confirmed to be significantly associated with increased HDL-C in another large-scale study of more than 100,000 subjects that will be discussed below [37]. Despite some technical difficulties in measuring \textit{EL} activity in human plasma, a number of assays have been developed, and data derived from them are consistent with the concept that reduced \textit{EL} activity is associated with increased HDL-C levels [82,83].

Cumulatively, evidence in cell lines, animals and humans – especially the human genetic data – makes a convincing case that an inverse relationship exists between \textit{EL} activity and HDL-C. These data strongly indicate that pharmacological inhibition of \textit{EL} would raise HDL-C – a hypothesis borne out by pre-clinical data in mice [70]. Based on the strong evidence base that reduced \textit{EL} activity causes increased HDL-C, there has been substantial interest in pharmacological inhibition of \textit{EL} as a therapeutic strategy to raise HDL-C.

However, the connection between \textit{EL} activity and atherosclerosis or cardiovascular risk is more uncertain. In addition to its effect on HDL-C levels, \textit{EL} has been shown to affect angiogenesis and vasorelaxation by affecting the binding of HDL-associated sphingosine-1-phosphate with endothelial cell receptors [84] and generating lysophosphatidylcholine species, respectively [85]. Atherosclerosis-prone \textit{Lipg}^{+/−}/\textit{Apoe}^{−/−} mice showed attenuated atherosclerosis when compared with mice in which apoE alone was deleted [86]. However, another group reported that similar \textit{Lipg}^{+/−}/\textit{Apoe}^{−/−} mice, as well as \textit{Lipg}^{+/−}/\textit{Ldlr}^{−/−} mice, had higher HDL-C but no difference in aortic atherosclerotic lesion areas compared with \textit{Lipg}^{+/+}/\textit{Apoe}^{−/−} and \textit{Lipg}^{+/+}/\textit{Ldlr}^{−/−} mice [87]. Thus, the effect of \textit{EL} deficiency on atherosclerosis in mice is uncertain.

Consequently, there has been substantial interest in determining whether genetic variation in \textit{EL} is associated with cardiovascular risk in humans. An association study of 17 common \textit{EL} SNPs (not including N396S) in a population of 3916 subjects found no significant associations with MI after correction for multiple testing [88]. A case–control study (described above) of 3375 subjects found no association between five separate common \textit{EL} SNPs (again not including N396S) and MI, but did note an association between three of them and deep vein thrombosis risk [75]. However, another study involving sequencing of
177 individuals with HDL-C levels at or above the 90th percentile identified six novel LIPG mutations, as well as the N396S variant and another known variant, R476W. The two known mutations were found to reduce in vitro EL activity by 50% (partial loss of function), while the newly identified rare variants showed complete loss of function, and significantly increased cholesterol efflux acceptor capacity. A meta-analysis of 15,496 subjects found a nonsignificant trend toward reduced coronary artery disease in carriers of the more frequent N396S and R476W variants [89].

The most convincing evidence to date against an association between EL and cardiovascular disease comes from a 2012 Mendelian randomization study that examined the frequency of the loss-of-function N396S variant in a collected population of 20,913 MI cases compared with 95,407 controls to determine whether partial genetic EL deficiency caused by this mutation was in fact cardioprotective [37]. As noted above, there was a highly significant increase in HDL-C in carriers of the N396S variant. Based on observational epidemiology, this increase in HDL-C, if causal, would be predicted to result in a concurrent 13% decrease in MI risk. However, the variant N396S allele was not associated with decreased risk of MI (odds ratio [OR]: 0.99; 95% CI: 0.88–1.11; p = 0.85). While the effect of heterozygous EL N396S on HDL-C levels is modest, thus perhaps making it harder to see clear benefit with regard to CAD, the number of subjects studied provided adequate power to detect even a small reduction in MI.

Cumulatively, these data call into question whether EL inhibition will decrease CHD risk, even though it would be fully expected to increase HDL-C levels. Although a series of molecular inhibitors of EL have been reported [90,91], none have been advanced into the clinic. Additional data, such as pharmacologic inhibition demonstrating reduced atherosclerosis in preclinical models and/or additional human genetic data with genetic instruments of greater effect, are necessary to give sufficient confidence in this mechanism as a therapeutic target.

**ApoC-III**

The cases of CETP and EL provide two lessons for the future of single-gene studies in HDL-C research. First, they emphasize that raising HDL-C levels alone does not necessarily confer reduced cardiovascular risk. Second, well-designed genetic studies can provide researchers with a great deal of information regarding the advisability of targeting a particular gene for pharmaceutical development. Study of a third gene of interest for HDL-C, APOC3, may provide an opportunity to apply these lessons.

ApoC-III was first isolated and shown to be a noncompetitive inhibitor of LPL in 1972 [92]. The gene encoding apoC-III, APOC3, was cloned 11 years later [93], and soon after was shown to be organized in tandem with APOA1, APOA4 and APOA5 [94]. Preclinical data examining the relationship between apoC-III and HDL-C levels are somewhat limited. ApoC-III overexpression in mice led to increased TG and decreased apoA-I and HDL-C [95]. Conversely, a study of APOC3 knockout mice found that they exhibited hypotriglyceridemia, although no significant changes in apoA-I or HDL-C levels were noted [96]. In a correlational study of 19 human subjects, HDL–apoC-III and HDL–apoA-I

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concentrations were positively correlated, while there was an inverse correlation between HDL–apoC-III concentration and apoA-I catabolism, hypothesized to be due to apoC-III's inhibition of HL in addition to LPL [97]. A more recent study in 39 men with central obesity and 12 non-obese controls similarly found that subjects with central obesity had elevated apoC-III and HDL–apoC-III concentrations, along with a reduced fractional catabolic rate of HDL–apoC-III [98]. However, it also noted that HDL–apoC-III concentration itself was in fact associated with hypercatabolism of HDL–apoA-I, implying that apoC-III may play a role in lowering HDL-C, perhaps due to an increase in unstable, TG-rich HDL particles, given the hypertriglyceridemia caused by increased apoC-III-mediated inhibition of LPL.

A number of studies examined plasma and lipoprotein apoC-III concentrations and concluded that they are directly related to cardiovascular risk. A series of nested case–control studies determined that apoC-III content of VLDL and LDL was a significant predictor of subsequent coronary events [99], and that LDL with apoC-III levels were a strong predictor of recurrent coronary events in a population that had already had an MI [100]. A prospective cohort study of 633 patients with pre-existing CHD noted that elevated apoC-III levels (≥10.5 mg/dl above the median) were significantly associated with both total and cardiovascular mortality over a 5-year period [101], while another study found that for 739 CHD-free subjects who developed CHD over 10–14 years of follow-up and matched controls, LDL–apoC-III levels were significantly associated with CHD risk [102].

A trial of 162 male subjects postcoronary artery bypass grafting found that HDL–apoC-III levels were the primary risk factor predicting coronary artery disease progression in a combination colestipol/niacin-treated group; subjects without disease progression had high HDL–apoC-III and lower levels of apoC-III in VLDL and LDL [103]. Another trial of 270 hyperlipidemic subjects randomized to lovastatin or placebo noted that VLDL-associated apoC-III was a risk factor for progression of pre-existing mild-to-moderate atherosclerotic lesions [104]. A third 63-patient clinical trial noted that coronary artery disease progression, as measured by coronary angiography in both statin- and placebo-treated groups, was associated with apoC-III levels, as well as levels of apoC-III on VLDL and LDL [105]. Another study of 100 men who had undergone coronary angiography noted that CAD severity correlated with apoC-III concentration in heparin precipitate [106]. However, an earlier study found that apoC-III content was not linked with CAD progression [107]. In addition, a recent pooled analysis of two nested case–control studies comprising 634 CHD cases and 637 controls found that HDL–apoC-III was associated with a significantly increased risk of CHD, while apoC-III-free HDL was associated with significantly decreased risk [108]. Another paper showed increased apoC-III content on HDL in subjects with CHD than in those without; furthermore, apoC-III was shown to impair the endothelial antiapoptotic function of HDL [109].

Several genetic studies have investigated the relationship between apoC-III and HDL-C. Two subjects with very high HDL-C and apoA-I levels, as well as greatly decreased apoC-III levels, were found to be heterozygous for a K58E mutation [110]. Heterozygous carriers of an 8017G>C noncoding polymorphism (also known as SstI) had higher plasma apoC-III and TG concentrations, as well as smaller LDL peak particle diameter in a study of 91 healthy subjects [111]. A study of 1030 Chinese subjects noted that the same SstI SNP was
more common in women, but in men was associated with increased HDL-C [112]. The gene
*GALNT2*, a locus linked with HDL-C levels [113], also warrants discussion. Its protein
product is hypothesized to activate apoC-III, rendering it functional; two individuals with
loss-of-function mutations in *GALNT2* were found to have elevated HDL-C and decreased
glycosylation of apoC-III [114].

Studies of common genetic variants at the *APOC3* locus have suggested an association with
CHD, although results are variable. One study found a weak, nonsignificant negative
association of an 1100C>T SNP in *APOC3* with atherosclerosis in patients who had suffered
MIs at a young age compared with age-matched controls [115]. The majority of studies have
examined the −455T>C, −482C>T and 8017G>C (SstI) noncoding SNPs that are in linkage
disequilibrium. Carriage of the minor allele at all three of these loci, termed the *APOC3*+222
haplotype, has been associated with increased MI risk compared with wild-type status at all
three loci in a case–control study of 3406 Costa Rican subjects [116]; however, a later study
in the same population found that this increased cardiovascular risk, as well as higher apoC-
III levels, were only seen in lean *APOC3*+222 haplotype carriers and those with low fasting
glucose. This effect was hypothesized to be due to insulin-mediated repression of apoC-III
expression in wild-type individuals but not in mutation carriers or in subjects with insulin
resistance [117]. Similarly, subjects homozygous for the −482C>T polymorphism in a study
of 519 Turkish individuals had higher apoC-III levels only if they had abdominal obesity or
insulin resistance; high circulating apoC-III levels were also noted to be predictive of CHD
in this population, although the particular SNP itself was not [118]. However, another
prospective cohort study of 498 subjects following high-throughput genotyping noted that
the −482C>T polymorphism was significantly associated with major cardiac events, such as
CHD-related death, nonfatal MI and revascularization [119]. In a study of 873 patients, 549
of whom had CHD and 270 had the metabolic syndrome, the −455T>C polymorphism by
itself was associated with higher apoC-III levels as well as CHD; furthermore, patients with
the metabolic syndrome were more likely to have increased apoC-III [120]. The same SNP
has also been associated with lower HDL-C and acute coronary syndrome in a population of
229 Han Chinese cases compared with 254 healthy controls [121], although another study of
286 Han Chinese CHD cases and 325 controls found no relationship between either the
−455T>C or −482C>T SNPs and CHD risk [122]. A study of 342 subjects with parental
history of MI compared with 114 controls noted significantly higher apoB levels, but failed
to find a difference in SstI polymorphisms between the two groups [123]. Another study in
which 102 Italian subjects with established CHD and 104 controls were followed for 8 years
found no association of the SstI locus with revascularization, MI or cardiovascular death
[124]. Similarly, a prospective nested case–control study of 385 MI cases and 373 controls
found no significant difference in SstI allele or genotype frequency between the two groups
[125]. Another population study of 158 CHD patients, 35 patients with <70% stenosis on
angiography and 151 CHD-free controls also failed to find any difference in SstI allele
frequency between cases and controls [126], while a study of 219 diabetic and nondiabetic
subjects found no association between SstI and either MI or diabetes [127]. However, a
study of 200 Egyptian MI patients compared with 100 controls found that the SstI
polymorphism was associated with a tripled risk of MI [128], and a larger study of 326 male
MI patients and 361 controls noted a significant association of the SNP with MI [129].
Rare and low-frequency nonsynonymous variants in APOC3 of greater effect may be more informative with regard to atherosclerosis. One study identified an APOC3 stopgain mutation (R19X) predicted to cause complete loss of protein function [130]. Approximately 5% of a population of 809 old order Amish subjects was found to be heterozygous for this variant. In a subset of 20 related subjects, heterozygotes had apoC-III levels less than half of those of non-carrier relatives (p = 0.0002). Analysis of the entire population found that carriers had higher HDL-C (67 vs 55 mg/dl; p = 9.0 × 10^-7) and lower LDL-C (116 vs 140 mg/dl; p = 0.02) and total cholesterol (191 vs 209 mg/dl; p = 0.001) when compared with noncarriers. Heterozygotes also had lower TG levels both when fasting as well as after an oral fat challenge. Importantly, coronary artery calcification scoring by CT scanning found that mutation carriers were much less likely to have detectable coronary artery calcification (OR: 0.35; 95% CI: 0.21–0.60), a marker of coronary atherosclerosis, or to have a score at or above their ethnicity-, sex- and age-specific 75th percentile (OR: 0.38; 95% CI: 0.19–0.77; p = 0.003). This finding was interpreted by the authors as indicative of a cardioprotective effect from partial apoC-III deficiency [130].

The low-frequency R19X mutation causing reduced apoC-III mass and activity is associated with increased HDL-C, as well as reduced TGs and LDL-C. This mutation also appears to be associated with reduced coronary atherosclerosis, although more data is required to confirm that this is the case. Taken together, these points suggest that therapy to reduce or inhibit apoC-III might not only raise HDL-C, but also reduce CHD risk. However, apoC-III – unlike CETP and EL – is a structural lipoprotein that may be difficult to inhibit utilizing a small-molecule approach. Consequently, attention has turned toward targeting apoC-III with biological approaches. One example, ISI-APOCIII_RX, is an apoC-III antisense oligonucleotide that has been studied as a potential intervention for Type 1 diabetes and shown to lower apoC-III levels in a rat model of that condition [131]. According to unpublished reports, the drug decreased apoC-III levels up to 78% in a Phase I study of 32 subjects and is currently undergoing a Phase II trial [203,204]. Similar gene silencing approaches have been carried out through the use of antisense oligonucleotides and RNAi directed against apoB-100 and PCSK9, as have been reviewed elsewhere [132].

**Conclusion**

Three monogenic causes of elevated HDL-C – CETP (encoded by CETP), EL (encoded by LIPG) and apoC-III (encoded by APOC3) – have received a great deal of attention over the past decade. Identification of these genes and analysis of rare genetic variants in them have allowed researchers to increase understanding of HDL metabolism significantly. However, development of related therapeutics to decrease cardiovascular risk has been less straightforward.

Despite the failure of two CETP inhibitors, the effect of potent CETP inhibition on cardiovascular end points is still unclear. The case of EL provides an example of the ability of population-level genetic studies to give information on cardiovascular disease end points. Finally, the mounting evidence that apoC-III levels are inversely related not only to HDL-C levels, but also to atherosclerosis and cardiovascular disease make it, unlike EL, a promising target for drug discovery.
The growing body of evidence that the clinical measure HDL-C does not reflect the functionality of HDL should guide further investigation into single genes thought to be relevant to HDL metabolism. Functionality measures such as cholesterol efflux potential – shown to be inversely related to CIMT and angiographic coronary atherosclerotic disease [133] – appear to be better metrics of a particular mutation's effect on reverse cholesterol transport and HDL-mediated athero protection than simple HDL-C levels. This investigational approach, when combined with studies of atherosclerosis and CHD, may prove fruitful for the investigation of other promising HDL-related genes, such as SCARB1, LIPC and LPL. Variation in SCARB1, encoding the HDL receptor scavenger receptor-BI, has been shown to lead to increased HDL-C in a population of 18 individuals heterozygous for a loss-of-function P297S mutation, although without obvious effect on carotid atherosclerosis [134]. Mutations in LIPC, which encodes HL, and in LPL, have been implicated in CHD in a recent Mendelian randomization study [135]; both genes warrant continued study.

The three genes discussed in this review illustrate both the potential benefits and pitfalls of single-gene studies in advancing HDL-related cardiovascular therapeutics. Such studies should continue to prove indispensable in advancing knowledge of the role HDL-C plays in reverse cholesterol transport and in identifying targets for drug development. In order to lead to efficacious pharmaceuticals, studies of newly identified genes of interest must focus not only on lipid levels such as HDL-C, but also on the effect of genetic variants on measures such as HDL functionality, atherosclerosis, stroke, MI and CHD-related death, as well as noncardiovascular outcomes.

Future perspective

Single-gene studies will continue to contribute important knowledge about HDL metabolism and potential therapeutic targets. The role of large-scale genetic studies in identifying promising genes and variants will grow in importance. Novel genes and mutations affecting HDL metabolism will continue to be identified through sequencing. Indepth phenotypic evaluations of subjects carrying these variants will enable careful characterization of their effects and potential utility as drug targets. In addition, Mendelian randomization studies will allow assessment of the relationship between these genes and CHD.

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Executive summary

Background

- Loss-of-function mutations in CETP, LIPG, and APOC3 (encoding CETP, endothelial lipase [EL], and apoC-III, respectively) are known monogenic causes of elevated HDL-cholesterol (HDL-C) levels.

CETP

- CETP activity correlates directly with LDL-cholesterol and inversely with HDL-C. Individuals with genetic CETP deficiency appear to have a modest reduction in coronary heart disease risk. However, clinical trials of the CETP inhibitors torcetrapib and dalcetrapib have failed to show a decrease in coronary heart disease incidence. Preclinical studies of CETP deficiency and CETP inhibitors alike may have been overly focused on seemingly favorable lipid profile changes instead of atherosclerotic burden and cardiovascular risk.

Endothelial lipase

- EL activity correlates inversely with HDL-C. However, animal studies have failed to conclusively show that decreased EL activity decreases atherosclerosis susceptibility. Furthermore, a large-scale genetic study did not show any association between a loss-of-function mutation in LIPG (N396S) and myocardial infarction risk, despite a substantial increase in HDL-C in mutation carriers. Development of pharmacological endothelial lipase inhibitors has slowed following publication of this study.

ApoC-III

- ApoC-III levels correlate directly with triglyceride levels and inversely with HDL-C. A number of preclinical and clinical studies convincingly show that apoC-III levels are also directly related with cardiovascular risk, that certain gain-of-function APOC3 variants are associated with major cardiac events and that a common loss-of-function variant (R19X) is cardioprotective. These findings have increased interest in targeting apoC-III in order to decrease atherosclerosis and CHD risk.

Conclusion

- These three cases illustrate the importance of single-gene studies to future translational research and drug development relating to HDL-C. Such studies must focus not only on mutations and medications that lead to favorable lipid profile changes, but also on measures of atherosclerosis and cardiovascular disease risk. Population-wide genetic studies have proven useful in attaining such data.