# Commentary on Cutting Edge Science

# Triglyceride-Rich Lipoproteins and Coronary Artery Disease Risk
## New Insights From Human Genetics

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Despite ample success in reducing coronary artery disease (CAD) risk through reduction of low-density lipoprotein cholesterol (LDL-C), there remains substantial residual risk.[1–4] Recent prospective studies have demonstrated that elevated triglycerides (TGs) are independent predictors of CAD risk.[5–9] Furthermore, TGs are strongly associated with incident CAD events in patients with low LDL-C levels treated with statin.[10] Thus, triglyceride-rich lipoproteins (TRLs) offer a potentially orthogonal risk factor to LDL-C for lowering CAD risk, but only if TRLs are causally associated with atherosclerotic disease.[11]

Human genetics has the potential to reveal the causal relationships of biomarkers found to be associated with disease outcomes.[12–15] For example, genetic variants associated with plasma LDL-C levels are consistently associated with CAD risk in the right direction,[15–18] consistent with a causal relationship. Importantly, similar studies have causally implicated the key TG-regulating enzyme lipoprotein lipase (LPL) in CAD risk. A common gain-of-function *LPL* variant, S447X, confers an antiatherogenic lipid profile characterized by low levels of TGs, and in several studies, it has been associated with lower incidence of vascular disease or myocardial infarction (MI).[19–25] Conversely, several loss-of-function (LOF) *LPL* variants associated with elevated TG levels have been reported to be associated with increased CAD risk.[21,26] Furthermore, multiple genome-wide association studies in the last 5 years have identified common noncoding variants at the *LPL* gene locus associated with both TG and CAD risk in the same direction.[27–29]

Beyond *LPL* itself, common variants that influence TG levels are significantly associated with CAD risk even after adjusting for their effects on other lipid traits.[30] Do et al[30] surveyed 185 single-nucleotide polymorphisms (SNPs) that were genome-wide significantly associated with ≥1 plasma lipid trait and identified a subset of 44 SNPs with large effects on TG levels but minimal effects on LDL-C. They tested the association of these SNPs with CAD in >86000 individuals. The strength of association of the SNPs with TG levels predicted the magnitude of association with CAD risk. Among the common variants with strong associations with both TG and CAD were those at a gene locus containing the genes *APOC3* and *APOA5*, which encode apolipoproteins (apoC-III and apoA-V, respectively), found on TRLs and known to be the regulators of LPL activity and TG levels.

ApoC-III is a key regulator of fasting and postprandial plasma TG levels and is thought to act at multiple nodes influencing TG homeostasis. A small (8.8 kDa) secreted apolipoprotein, apoC-III, is expressed in the liver and intestine and circulates on and exchanges between TRLs and high-density lipoprotein (HDL).[31,32] Several studies have suggested that apoC-III negatively regulates LPL activity.[33] Further insight gained from transgenic mice overexpressing *APOC3* and *Apoc3* knockout mice has shown that apoC-III delays very LDL (VLDL)–TG hydrolysis in vivo and may delay the catabolism of TRL remnants by the liver and other tissues.[34–37] In addition, 1 human coding variant in *APOC3*, K78E, is associated with low TG and high HDL-C levels and was shown to reduce VLDL secretion in vivo.[38] This suggests that *APOC3* may contribute to plasma lipids at least in part through influencing hepatic VLDL assembly and secretion.

Like apoC-III, apoA-V is also an exchangeable apolipoprotein between HDLs and TRLs, which is primarily secreted from the liver. It is a 39-kDa protein and has a low concentration in human plasma (≈150 ng/mL)[39] compared with the major apolipoproteins, including apoC-III. Despite its low abundance, apoA-V is thought to play a crucial role in TG metabolism. *Apoa5* knockout mice demonstrate profound hypertriglyceridemia, whereas human *APOA5* transgenic mice have significantly lower plasma TG than controls.[40] ApoA-V has been shown to enhance LPL activity on VLDL particles. Recent work has suggested that it may do so by facilitating proximity between TRLs and LPL in part through apoA-V's interaction with glycosylphosphatidylinositol-anchored HDL binding protein 1, a chaperone for LPL. Like apoC-III, apoA-V may have a critical intracellular role in regulating TG metabolism as well. Numerous studies in cultured hepatocyte-like cell lines have suggested that apoA-V accumulates in the endoplasmic reticulum after translation and remains associated with hepatic lipid droplets.[39]

## Sequencing Reveals *APOC3* and *APOA5* as Causal Mediators of CAD Risk

Two recent reports, published concurrently in the *New England Journal of Medicine*, used complementary approaches to demonstrate that LOF mutations in *APOC3* are

robustly associated with lower TG and decreased incidence of CAD.[41,42] One of these studies, a large collaboration of the Exome Sequencing Project of the National Heart, Lung, and Blood Institute, sequenced the exomes of 3734 subjects and tested the association of identified mutations, either individually or in aggregate within a gene, that were associated with plasma TG.[41] They identified 7 coding variants in *APOC3* in a total of 33 individuals. Of these variants, all were rare in frequency, with 3 missense, 1 nonsense, and 3 splice-site variants identified. When tested in aggregate, the *APOC3* variants were robustly associated with lower TG (by 39 mg/dL) relative to noncarriers. Four of the 7 variants were found in heterozygosity at an aggregate frequency of 1 in 150 in individuals of European descent. These variants were associated with approximately half of the circulating apoC-III concentrations of noncarriers, supporting the notion that these variants conferred the loss of apoC-III function. The authors tested the association of these 4 variants with the presence of CAD in >110 000 subjects and found 40% lower CAD risk in mutation carriers. Notably, 1 of the 4 variants studied by Crosby et al,[41] a nonsense mutation R19X (rs76353203) was previously shown in an Amish population to reduce TG and improve the clearance of dietary fat in an oral fat challenge and was associated with reduced coronary artery calcium scores, a surrogate measurement of atherosclerosis.[43]

Working independently in Denmark, Jørgensen et al[42] tested the association of plasma TG with the presence of ischemic vascular disease (CAD or cerebrovascular disease) in 2 prospective cohorts comprising 75 725 subjects.[21] They found that the subjects with TG <90 mg/dL had significantly lower risk of ischemic vascular disease compared with the subjects with TG >350 mg/dL. Initial deep medical resequencing of the exons of *APOC3* and subsequent genotyping in the larger cohort identified 260 heterozygous carriers for 1 of 3 *APOC3* mutations, which were associated with lower fasting TG. The 3 variants identified by this approach were among the 4 SNPs that drove the association of *APOC3* variants with TG described by the Exome Sequencing Project of National Heart, Lung, and Blood Institute. Of the 75 725 subjects studied, 10 797 subjects developed ischemic vascular disease, of which 7557 had ischemic heart disease. When separated by *APOC3* genotype, they noted a 41% reduction in risk of vascular disease among mutation carriers. The association of the variants with lower incidence of vascular disease was attenuated when comparisons were adjusted for nonfasting TG levels in the participants, implying that the effect of apoC-III on TG levels is at least partially responsible for the protection from disease conferred by the variants.

These 2 recent studies present the argument that apoC-III's influence on plasma TG is responsible for the relationship of apoC-III with CAD risk. However, given apoC-III's pleiotropic influence on lipoprotein metabolism and additional contributions to vascular risk, others have suggested that this interpretation may be incomplete. Cohen et al[44] recently commented on the possibility that the reduced LDL-C levels in *APOC3* mutation carriers may account for the observed protection from vascular disease. ApoC-III on intermediate-density lipoproteins and LDLs is thought to delay hepatic clearance of these particles by lipoprotein receptors, and LDL-containing apoC-III was shown to be positively associated with development of coronary heart disease.[34,35,45] In addition, LDL-bound apoC-III was shown to be associated with levels of the proatherogenic small dense LDL independently of plasma TG.[46] For these reasons, deeper mechanistic studies in humans carrying these variants are warranted to explore the exact contribution(s) attributable to *APOC3* LOF that confers protection from vascular risk. These studies will undoubtedly require an isolated study of the specific candidate processes influenced by apoC-III in carriers versus noncarriers of the identified mutations.

In contrast to these studies identifying disease-protective *APOC3* LOF coding variants, studies of *APOA5* have revealed several risk-conferring LOF coding variants. Several coding variants have been implicated in severe hypertriglyceridemia or hyperchylomicronemia through case-control and family-based sequencing studies.[47] Many of these studies identified rare variants in *APOA5* but demonstrated that they were robustly associated with hypertriglyceridemic phenotypes when considered in aggregate.[47,48] In addition, some common coding variants in *APOA5* associated with increased TG have also been attributed to increased CAD risk.[49,50]

In December 2014, investigators from the Broad Institute reported in *Nature* a large exome sequencing experiment in early-onset MI cases compared with older healthy controls that implicated *APOA5*.[51] To test the hypothesis that rare alleles may contribute to the extreme phenotype of early-onset MI, Do et al[51] performed exome sequencing in a discovery cohort of 1027 early MI cases (men, ≤50 years old and women, ≤ 60 years old) and 946 older controls without MI (men, ≥60 years old and women, ≥70 years old) through participation in the Exome Sequencing Project of National Heart, Lung, and Blood Institute, selecting subjects from a total of 11 studies. In assessing the results of this sequencing effort, the authors collapsed rare variants in the same gene and tested their aggregate frequency within a given gene between cases and controls (gene-burden testing).[52,53] They compared the collective variants within each gene between cases and controls by 3 metrics of variant annotation: nonsynonymous variants without functional annotation, deleterious variants as identified by the prediction tool PolyPhen2-HumDiv, and disruptive (indel, frameshift, nonsense, and splice-site) variants only. This preliminary effort did not identify any variants studied collectively that were associated with MI when using a significance threshold of $P=8\times10^{-7}$, a conservative limit to account for testing ≈20 000 genes by 3 different variant classification schemes.

On expansion of the exome sequencing effort from 1973 to 9793 participants (4703 MI cases and 5090 controls), the investigators performed gene-burden testing again and found that rare alleles in the *LDLR* were significantly associated with the risk of MI. In total, they identified 285 *LDLR* variants in cases compared with 208 in controls, resulting in an effect size of 1.5 fold ($P=4\times10^{-6}$). To comprehensively filter the identified variants to yield the mostly likely functional ones for association testing with MI, the authors developed 5 sets of criteria based on combinations of existing coding variant prediction

tools and applied each set of criteria to the identified variants. After applying the more stringent annotation criteria sets, the authors found an even greater enrichment of rare *LDLR* variants in MI cases, with an effect size of 13 fold when only variants considered to be most disruptive were included ($9 \times 10^{-5}$). A total of 156 unique nonsynonymous coding, splice-site, and frameshift variants within LDLR were identified, of which 77 were previously reported as underlying causes of familial hypercholesterolemia, suggesting that the identified variants could cause MI through disruption of LDLR function and subsequent LDL-C elevation. This study provides hypothesis-free support for the well-established observation that genetically elevated LDL-C levels are frequently associated with increased risk of early MI.[54,55] Further functional study of the remaining 79 novel, rare *LDLR* variants will be required to determine whether and how they disrupt *LDLR* gene function and cause familial hypercholesterolemia.

The second major finding of this study was borne from additional targeted sequencing of 6 candidate genes (*APOA5*, *CHRM5*, *SMG7*, *LYRM1*, *APOC3*, and *NBEAL1*) that were identified as nominally significant ($P<0.005$) in the initial exome sequencing discovery phase. The coding regions of these 6 candidate genes were initially resequenced in 2 independent cohorts, 1 Italian cohort comprising 1716 early MI cases and 1519 controls, and another cohort from Ottawa consisting of 552 early MI cases and 586 controls. These initial efforts revealed an enrichment of rare *APOA5* mutations in early MI cases, prompting further sequencing of this gene in additional cohorts. Overall, sequencing of *APOA5* in 6721 early MI cases and 6711 MI-free controls identified 46 individual rare single-nucleotide variations. These variants were identified in 93 MI cases versus 42 healthy controls, and the >2-fold risk of MI in mutation carriers was primarily driven by variants found in only 1 or 2 study participants (private or near-private variants). Application of each of the 5 sets of variant annotation criteria for deleteriousness demonstrated a significant enrichment of rare *APOA5* alleles in MI cases, with greater relative risk of MI in individuals harboring variants deemed more deleterious by stricter criteria sets (strict and disruptive criteria).

The plasma lipids of harmful *APOA5* mutation carriers are important especially in light of the findings from the authors' previous study of CAD-protective *APOC3* variants associated with plasma TG. *APOA5* mutation carriers in the exome sequencing study had 63 mg/dL higher fasting TG and 14 mg/dL lower HDL-C than noncarriers, but notably, plasma LDL-C was comparable between carriers and noncarriers. These findings suggest that disruption of *APOA5* gene function increases the risk of CAD/MI through a mechanism that increases TRLs but does not involve alteration of LDL levels and provides further support to the previous evidence implicating genetically elevated TRLs in the risk of CAD/MI.

## Targeting the LPL Pathway to Reduce CAD Risk

These recent studies have provided powerful evidence that plasma levels of TRLs are causally related to the development of CAD and specifically that apoC-III promotes and apoA-V protects against CAD. The results of Do et al[51] implicating

*APOA5* LOF with increased TGs, no elevation in LDL-C, but increased MI risk also adds credence to the concept that it is the reduction in TRLs that primarily drives the association of the *APOC3* variants with reduced CAD/vascular disease incidence. Collectively, these 3 studies thus offer strong support to the hypothesis that intervention to lower TRL levels may decrease the risk of CAD. Taken together with previous investigations, they implicate the LPL pathway as a potential target for reducing the risk of CAD through modulation of TRL metabolism. Translating these findings to tangible therapeutic strategies will undoubtedly necessitate a better understanding of how the LPL pathway and its regulators, such as apoC-III and apoA-V, actually work in concert to regulate this metabolism.

In the case of apoC-III, 1 therapy to reduce its production is already in clinical development. ISIS Pharmaceuticals has developed an antisense oligonucleotide that silences *APOC3* expression in the liver.[56] This small chemically modified oligonucleotide is delivered subcutaneously and is internalized in the liver where it inhibits the translation of *APOC3* mRNA and promotes mRNA degradation through activation of RNase H. This anti-*APOC3* antisense oligonucleotide has been reported to significantly reduce plasma apoC-III and TG levels and blunt postprandial TG elevations on treatment of rodent models and a nonhuman primate model with anti-*APOC3* antisense oligonucleotide and in healthy human volunteers.[56] In December 2014, this anti-*APOC3* antisense oligonucleotide was reported to reduce TG levels in 3 patients with familial chylomicronemia.[57] Based on the human genetics, the expectation is that intervention to reduce plasma apoC-III levels will not only reduce TG levels but also decrease the risk of CAD.

Alternative approaches to targeting apoC-III will benefit from better structural elucidation of the apoC-III protein and mechanistic insights into the effects of the disease-protective variants identified. Although 3 of the 4 *APOC3* variants mainly responsible for the robust association with lower TG and CAD risk putatively function through affecting the production of full-length apoC-III protein (2 splice-site and 1 nonsense variant), the fourth variant, A43T (rs147210663), is a missense variant. This suggests that the variant may alter apoC-III function in a manner to render it less effective in maintaining plasma TG. A previous study of the biochemical properties of this variant suggested that it may alter lipid binding and thus may influence the exchangeability of apoC-III among lipoproteins or stability in circulation.[58] Further insight into apoC-III structure and lipid-binding properties, and the specific effects of such missense variants on these properties, may aid the development of small molecules or other structure-guided therapeutics that target a defined property of apoC-III function.

Development of treatments focused on enhancing the activity of apoA-V is conceptually more difficult to envision. Nevertheless, any efforts to do this will also benefit from careful structure–function studies of the lipid- and lipoprotein-binding properties of this protein. Given the low plasma concentration of apoA-V, studies of its structure–function relationships may offer insight into domains that could be modulated to increase binding affinity for VLDLs, promote retention or increased stability of the protein in a lipid-bound state. Interestingly, among the *APOA5* variants identified by exome sequencing,
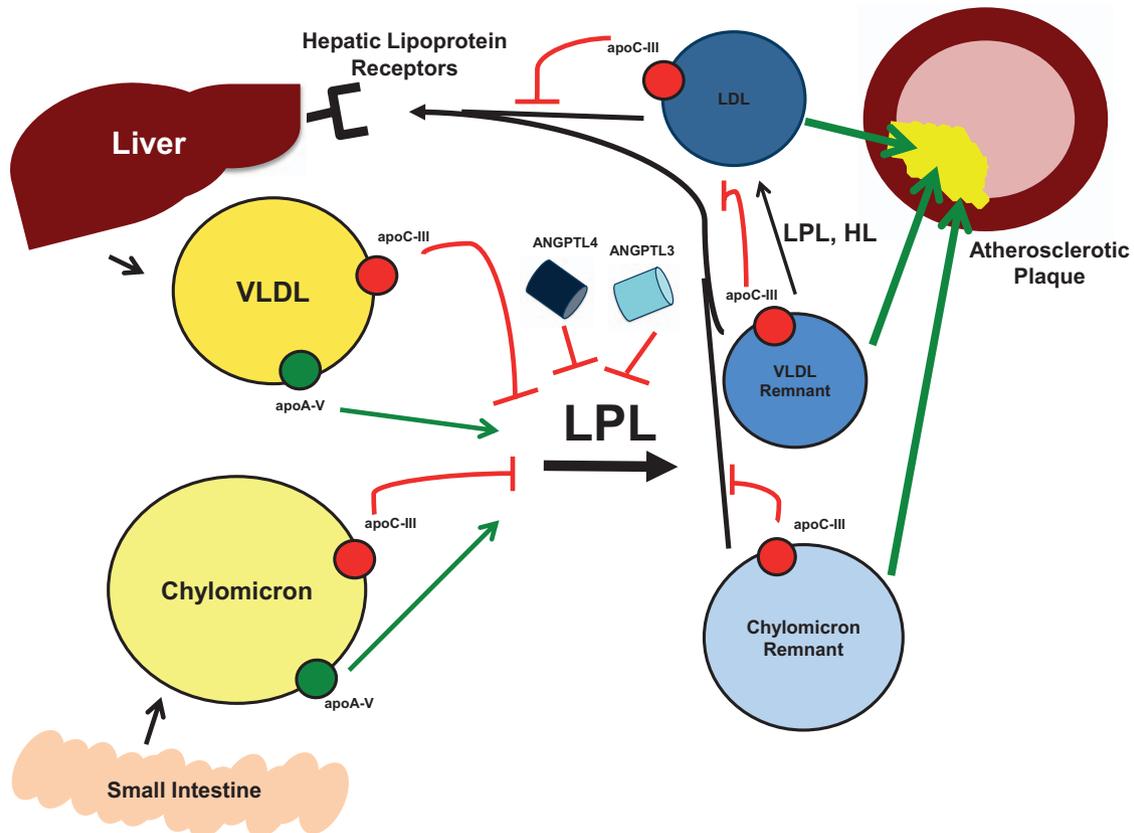
**Figure.** Roles of apoC-III and apoA-V in plasma triglyceride (TG) metabolism. ApoC-III and apoA-V are both found on TG-rich lipoprotein particles, such as very low-density lipoprotein cholesterol (VLDL) and chylomicrons, which are synthesized and secreted from the liver or intestinal enterocytes, respectively. ApoC-III inhibits lipoprotein lipase (LPL) activity, as do other secreted proteins ANGPTL3 and ANGPTL4. In contrast, apoA-V activates LPL activity. LPL-mediated TG lipolysis subsequently results in VLDL remnants from VLDL or chylomicron remnant particles derived from chylomicrons, both of which are enriched in cholesterol relative to TG. These particles may be taken up by the liver through interaction with specific lipoprotein receptors (VLDLR, LDLR, LRP1, and others), a process which is inhibited by apoC-III. VLDL remnants can be further modified by LPL and hepatic lipase (HL) to result in cholesterol-enriched LDL particles. If not removed from circulation, these various types of triglyceride-rich lipoproteins may be taken up by macrophages in the arterial wall, where they may contribute to vascular inflammation and atherosclerosis.

2 nonsynonymous missense variants were among those predicted to be deleterious by all 5 prediction algorithms used. These variants, Arg289Cys and Arg343Cys, both occur in the C-terminus of apoA-V, a region previously implicated as critical for lipid binding.[59] Further insight on apoA-V's structural composition and function will undoubtedly be gleaned from careful study of the most functionally deleterious coding variants identified from the exome and targeted sequencing. Such investigations are already underway; for example Sharma et al[60] demonstrated in the October 2014 issue of *ATVB* that 1 of the identified *APOA5* variants, Gly185Cys, disrupts apoA-V function by promoting aberrant disulfide bond formation of the mutant protein. This work combined the study of the variant through viral vector–mediated expression in *Apoa5* knockout mice with biochemical characterization of apoA-V from the plasma human carriers of the mutation. Such synergistic approaches will be necessarily to fully understand the implications of the many novel mutations identified and relate them to the physiology of plasma TG turnover.

These recent studies also raise interest in the prospect of targeting other regulators of LPL-mediated TG metabolism, including the angiopoietin-like (ANGPTL) proteins ANGPTL3 and ANGPTL4.[61] Like apoC-III, ANGPTL3 and ANGPTL4 are thought to inhibit LPL activity, leading to elevated plasma TG levels, although their respective mechanisms conferring LPL inhibition may be distinct.[61–66] Both common and rare variants in *ANGPTL3* are associated with plasma TG levels, and rare *ANGPTL3* LOF mutations underlie the Mendelian condition familial combined hypolipidemia, characterized by low plasma TG in addition to other lipid classes.[66–69] Although common variants at the *ANGPTL4* locus are associated primarily with HDL-C levels, rare coding mutations are robustly associated with reduced plasma TG.[66–68,70] These findings taken together suggest that pharmacological inhibition of these ANGPTLs could reduce plasma TGs by a mechanism similar to that of anti-*APOC3* focused therapies and result in reduced CAD risk. However, unlike the clear directional association of the *APOC3* variants to TG and CAD risk in the 2 *New England Journal of Medicine* studies, the evidence linking *ANGPTL3* and *ANGPTL4* LOF to CAD risk has been smaller or inconsistent.[71–74] The viability of targeting the ANGPTLs to reduce the risk of CAD will thus depend on both larger human genetics studies of clear LOF variants and better understanding of the physiological interplay of these proteins with different lipoprotein subclasses.

In summary, a remarkable confluence of robust human genetics findings for the past 6 months has convincingly and causally implicated triglycerides and TG-rich lipoproteins in the development of cardiovascular risk. Specifically, the LPL pathway and its reciprocal regulators apoC-III and apoA-V have been found to have a remarkably important influence on the risk of CAD (Figure). TRLs can be added to the list of apoB-containing lipoproteins, joining LDL and Lp(a), as causal risk factors of CAD. Whether novel interventions to reduce plasma levels of TRLs will be orthogonal and additive to LDL reduction in reducing cardiovascular risk remains to be established.

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## Disclosures

None.

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