Antisense Oligonucleotide Inhibition of Apolipoprotein C-III Reduces Plasma Triglycerides in Rodents, Nonhuman Primates, and Humans


**Rationale:** Elevated plasma triglyceride levels have been recognized as a risk factor for the development of coronary heart disease. Apolipoprotein C-III (apoC-III) represents both an independent risk factor and a key regulatory factor of plasma triglyceride concentrations. Furthermore, elevated apoC-III levels have been associated with metabolic syndrome and type 2 diabetes mellitus. To date, no selective apoC-III therapeutic agent has been evaluated in the clinic.

**Objective:** To test the hypothesis that selective inhibition of apoC-III with antisense drugs in preclinical models and in healthy volunteers would reduce plasma apoC-III and triglyceride levels.

**Methods and Results:** Rodent- and human-specific second-generation antisense oligonucleotides were identified and evaluated in preclinical models, including rats, mice, human apoC-III transgenic mice, and nonhuman primates. We demonstrated the selective reduction of both apoC-III and triglyceride in all preclinical pharmacological evaluations. We also showed that inhibition of apoC-III was well tolerated and not associated with increased liver triglyceride deposition or hepatotoxicity. A double-blind, placebo-controlled, phase I clinical study was performed in healthy subjects. Administration of the human apoC-III antisense drug resulted in dose-dependent reductions in plasma apoC-III, concomitant lowering of triglyceride levels, and produced no clinically meaningful signals in the safety evaluations.

**Conclusions:** Antisense inhibition of apoC-III in preclinical models and in a phase I clinical trial with healthy subjects produced potent, selective reductions in plasma apoC-III and triglyceride, 2 known risk factors for cardiovascular disease. This compelling pharmacological profile supports further clinical investigations in hypertriglyceridemic subjects. (*Circ Res. 2013;112:1479-1490.)*

**Key Words:** antisense oligonucleotides ■ apolipoprotein ■ apolipoprotein C-III ■ clinical trial ■ lipids and lipoproteins ■ pharmacology ■ triglycerides

Hypertriglyceridemia has been recognized by the National Cholesterol Education Program Adult Treatment Panel III as an independent risk factor for the development of coronary heart disease (CHD). Recent epidemiological studies and meta-analyses have reaffirmed that elevated triglyceride levels are associated with CHD and a greater risk of disease recurrence in patients with stable CHD. Furthermore, CHD risk is ameliorated when triglyceride levels are reduced.

Very high plasma triglyceride levels (>5.7 mmol/L [500 mg/dL]) are also associated with pancreatitis and may account for as much as 10% of all cases of routine acute pancreatitis and 50% of gestational pancreatitis. Although the risk of pancreatitis is considered significant in any patient with plasma triglyceride levels >11.3 mmol/L (1000 mg/dL), there is also an increased probability of developing the disease associated with even more modest levels. The pathogenesis of pancreatitis is still incompletely understood, but thought to be related to the proinflammatory state produced when triglyceride are metabolized by pancreatic lipases. Interestingly, elevated triglyceride levels seem to increase the incidence of pancreatitis, but not the severity of attacks.

Plasma triglyceride are complex lipids primarily transported on very low–density lipoprotein (VLDL) particles and chylomicrons that are synthesized in the liver and intestine, respectively. The plasma triglyceride concentration is a complex polygenic trait, but a variety of genetic determinants have been identified, including apolipoprotein C-III (apoC-III),
lipoprotein lipase, and a number of other genes. ApoC-III genetic variants that enhance apoC-III plasma concentrations were associated with higher plasma triglyceride and an increase in the incidence of nonalcoholic fatty liver disease. Conversely, variants that suppress apoC-III levels, as observed in a group of Old Order Amish subjects, were associated with lower triglyceride levels. Similar observations have been made with regard to lipoprotein lipase genetic mutations. ApoC-III, a key regulator of plasma triglyceride levels, is a 79-aa glycoprotein, synthesized principally in the liver and to a lesser extent, by the intestine. Multiple apoC-III protein molecules reside on the surface of apoB-containing lipoproteins and high-density lipoproteins (HDLs), a percentage of which exchange rapidly between these particles. The apoC gene is located within the apolipoprotein A-I and apolipoprotein A-IV gene cluster on chromosome 11q23. ApoC-III expression is suppressed by insulin and is induced by glucose via transcriptional regulatory elements in the gene promoter region. Activation of peroxisome proliferator–activated receptor-α also reduces apoC-III expression, accounting in part for the hypotriglyceridemic action of fibrates. Elevated plasma apoC-III protein possesses several proatherogenic properties. ApoC-III is a potent inhibitor of the lipolysis of triglyceride-rich lipoproteins by antagonizing apolipoprotein C-II activation of lipoprotein lipase and hepatic lipase, which play an important role in both the conversion of VLDL to intermediate-density lipoproteins to low-density lipoprotein (LDL) and in the remodeling of HDL. It has also been suggested that apoC-III regulates apoB lipoprotein in apoB lipoprotein metabolism by promoting intrahepatic VLDL assembly and secretion, reducing triglyceride-rich lipoprotein clearance, and increasing the formation of atherogenic small dense LDL. Additionally, the enrichment of HDL with apoC-III may render a normally protective molecule atherogenic. There is also evidence that apoC-III promotes inflammation and endothelial cell dysfunction by enhancing monocyte cell adhesion via increased vascular cell adhesion molecule-1 expression. Finally, apoC-III levels are increased in type 1 diabetic patients and are also thought to be a cofactor in pancreatic β cell death. Taken together, these results support the concept that apoC-III is a multifunctional protein that not only regulates the metabolism of triglyceride-rich lipoproteins, but may also contribute to CHD and pathophysiological metabolic states.

In most, but not all, studies plasma apoC-III levels have been positively associated with CHD risk. In mouse models, genetic ablation of apoC-III had no effect on atherosclerosis; however, overexpression of human apoC-III in the low density lipoprotein receptor (Ldlr)−/− background significantly increased atherosclerosis. In humans, it was recently reported in a prospective study analysis that the risk for fatal or nonfatal myocardial infarction was significantly increased in subjects with apoC-III containing VLDL and LDL. Furthermore, the genetic variants that suppress plasma apoC-III levels in the Old Order Amish subjects and Ashkenazi Jew populations also exhibited reduced risk of CHD.

Although a variety of therapeutic agents reduce triglyceride levels (statins, niacin, fibrates, and Ω-3 fatty acids), some patients still cannot meet their recommended triglyceride goals, suggesting a need for more effective drugs. Therefore, we postulated that a direct inhibitor of apoC-III, itself an independent CHD risk factor, might provide therapeutic benefit because of its broad regulatory effects on triglyceride, triglyceride-rich lipoproteins, and HDL particle homeostasis. In this article, we describe the identification and characterization of a second-generation antisense drug that selectively reduces apoC-III in transgenic mice, nonhuman primates, and healthy human volunteers. We demonstrate the selective dose-dependent reduction of apoC-III with concomitant triglyceride lowering in multiple preclinical models and species, and in humans. Importantly, we also show that apoC-III reduction is well tolerated and is not associated with increased liver triglyceride accumulation or hepatotoxicity. These results support the advancement of the human apoC-III drug to phase 2 investigations in patients with hypertriglyceridemia.

Methods

An expanded methods section can be found in the online Data Supplement Material.

Oligonucleotides

A series of chimeric 20-mer phosphorothioate antisense oligonucleotides (ASOs) containing 2′-O-methoxyethyl groups at positions 1 to 5 and 16 to 20 targeted to murine, rat, and human apoC-III mRNA, as well as a control ASO, were synthesized and purified on an automated DNA synthesizer using phosphoramidite chemistry as previously described.

Preclinical Pharmacology Models

An institutional animal care and use committee approved all procedures and protocols for the preclinical pharmacology studies. See the expanded online Data Supplement Material section for detailed descriptions of the ASO sequences, animal strains/models, and of all experiments.

Phase I Clinical Trial in Healthy Human Volunteers

A randomized, placebo-controlled, double-blind, ascending dose, phase 1 study was conducted in healthy volunteers to evaluate the safety, pharmacokinetics, and pharmacological effects of the human apoC-III ASO, ISIS 308401, in humans. The study protocol was approved by a central institutional review board (Institutional Review Board Services, Canada) and performed in compliance with the standards of good clinical practice and the Declaration of Helsinki in its revised edition. See Online Figure I for diagrams of the single and multiple dose cohort schedules, Online Figure II for the flow of participants through the study, and Online Table I for baseline characteristics of subjects assigned to the multiple dose cohorts.

Results

Identification of ISIS 304801 (Human ApoC-III ASO) and Rodent-Specific ApoC-III ASOs

The apoC3 gene, which is conserved in eukaryotes, is 500 base pairs in length, containing 3 introns and 4 exons. The human, rhesus monkey, and cynomolgus monkey
genes are highly conserved with ≈93% homology. To identify potential human candidates, ≈350 second-generation 2′-O-methoxymethyl chimeric ASOs were screened against ≈200 sites (Online Figure III). All the ASOs were 18 to 20 nucleotides long, and the majority were of the 5-10-5 design, that is, five 2′-O-methoxymethyl nucleotides at the 5′ end, 10 deoxynucleotides in the center, five 2′-O-methoxymethyl nucleotides at the 3′ end, and phosphorothioate substitution throughout.46 Active ASOs were further evaluated in dose–response studies. Microwalks around sites where active ASOs were identified were performed to further delineate the selection of lead candidates. Throughout the process, the use of appropriate control ASOs assured that all of the compounds were highly selective.

The 26 most potent human ASOs were evaluated in human apoC3 transgenic mice and in a 4-week tolerability study in mice at a high dose (100 mg/kg per week). Based on its significant, dose-dependent reductions in human apoC-III mRNA, protein, and triglyceride in the transgenic model (Figure 1) and an attractive tolerability profile in mice (data not shown), ISIS 308401 was selected for further evaluation in nonhuman primates and the clinic. A similar process involving in vitro and in vivo evaluations47 was used to identify the mouse-(ISIS 440726) and rat-specific (ISIS 353982) apoC-III ASOs.

**ApoC-III ASOs Reduce ApoC-III mRNA and Triglyceride in Rodent Preclinical Models**

To fully explore the spectrum of apoC-III ASO pharmacological effects as a function of different dyslipidemic states, we evaluated a variety of mouse and rat strains, diets, and disease models. Species-specific apoC-III ASOs, identified through the process described above, were administered once weekly over 6 weeks to C57BL/6, Ldlr−/−, ob/ob, and cholesteryl ester transfer protein (CETP) transgenic / Ldlr−/− mice, as well as the fructose-fed and Zucker diabetic fatty rat models. As shown in Table 1, hepatic apoC-III mRNA was reduced by 66% to 98% in all treated animals, with the greatest reduction being observed in CETP transgenic, Ldlr−/− mice. Consistent with effects on apoC-III mRNA, fasting triglyceride levels were suppressed by 19% to 89%, with absolute reductions varying depending on the model and diet. Interestingly, perhaps because of its expression of CETP, the CETP transgenic Ldlr−/− mice were the only rodent preclinical model that showed increases in HDL-cholesterol (Online Table VII). More detailed pharmacological and mechanistic studies were performed in C57BL/6 and Ldlr−/− mice fed either normal chow or a Western diet (WD), and the data will be described below.

ISIS 440726, a mouse-specific apoC-III ASO (3.1, 6.3, and 12.5 mg/kg per week) and a control ASO (12.5 mg/kg per week) were administered by intraperitoneal injections to wild-type C57BL/6 mice fed either normal chow or a WD for 6 weeks. At the end of treatment, mice were fasted for 5 hours, euthanized, and liver apoC-III mRNA, plasma protein, and other parameters were evaluated (Online Table II). ISIS 440726 produced dose-dependent reductions in hepatic apoC-III mRNA and plasma protein in mice fed either diet (Figure 2A and 2B). Significant reductions in triglyceride were produced at the 12.5 mg/kg per week dose in mice fed normal chow (19%) and at all doses in the WD-fed animals (37%, 50%, and 44% in the 3.1, 6.3, and 12.5 mg/kg per week groups, respectively, Online Table II). Fast protein liquid chromatography analysis demonstrated that the triglyceride loss was primarily in the VLDL fraction (Online Figure VIA). Postprandial triglyceride were also reduced by ≈30% (Figure 2C and 2D). This change in postprandial triglyceride was not because of differences in intestinal triglyceride absorption, because after a poloxamer 407 block and oral gavage of 3H-triolein, appearance of 3H radioactivity in plasma was not different between control ASO and apoC-III ASO-treated mice (Figure 2E). To demonstrate that the activities of the apoC-III ASO resulted from target-specific inhibition, the pharmacology of the murine-specific ASO was compared in wild-type chow-fed C57BL/6 and apoC-III−/− mice. Six weeks of treatment with the mouse apoC-III ASO (12.5 mg/kg per week) reduced liver apoC-III mRNA to levels similar to those observed in the whole body apoC3−/− mice (data not shown). ISIS 440726 significantly (P<0.05) reduced fasting plasma triglyceride from 0.95±0.06 mmol/L (84±5 mg/dL) in saline controls to 0.79 mmol/L (70.3 mg/dL) in the wild-type mice. Treatment of apoC-III−/− mice with the apoC-III ASO failed to produce reductions in fasting plasma

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**Figure 1.** Representative experiment showing dose-dependent reduction in human apolipoprotein C-III (apoC-III) mRNA, plasma apoC-III protein, and triglyceride (TG) in mice containing the human apoC3 transgene after administration of ISIS 304801, the human apoC-III antisense oligonucleotide (ASO). ISIS 304801 was administered once weekly at 50, 15, and 1.5 mg/kg per week (n=3 per group) via intraperitoneal injection for 2 weeks. Data in A present apoC-III plasma protein (mg/dL) and triglyceride (mmol/L) levels in absolute concentration ±SD. *Significant difference from saline cohort using 1-way ANOVA post hoc Tukey multicomparison test (P<0.05).
triglyceride (0.64±0.08 mmol/L [56±7 mg/dL] in saline versus 0.70±0.01 mmol/L [62±1 mg/dL]), supporting the notion that the pharmacological effects observed in other mouse models were attributable to reductions in apoC-III protein.

Because previous publications suggested that apoC-III plays a role in VLDL secretion, we next assessed the effects of apoC-III reduction on VLDL and triglyceride export from the livers of chow- and WD-fed C67BL/6 and apoC3−/− mice administered 12.5 mg/kg/wk for 6 weeks.48 Secretion of triglyceride, as assessed by a poloxamer 407 block, was unaffected (data not shown). We also evaluated the effects of the mouse-specific apoC-III ASO on apoB-containing lipoprotein particle size and demonstrated that there was a reduction in large VLDL and chylomicron particles and a modest reduction in small VLDL particles (Figure 4B).

ISIS 304801 (Human ApoC-III ASO) Reduces ApoC-III mRNA, Protein, and Triglyceride in Chow-Fed Cynomolgus and Hypertriglyceridemic Rhesus Monkeys

Because the binding site for the human apoC-III ASO, ISIS 304801, is 100% conserved between cynomolgus and rhesus monkeys, and lipid metabolism in nonhuman primates resembles that observed in humans, we evaluated the effects of that drug on hepatic apoC-III mRNA, plasma apoC-III protein, and triglyceride in monkeys. When a 13-week pilot study (data not shown) was performed in chow-fed cynomolgus monkeys, ISIS 304801 (administered at doses of 4, 8, 12, and 40 mg/kg per week) reduced apoC-III mRNA in a dose-responsive fashion by 47%, 51%, 80%, and 89%, respectively, with a calculated ED50 for apoC-III protein of 6.3 mg/kg per week. Because these animals had very low triglyceride levels, another study was performed in a rhesus monkey model of hypertriglyceridemia. Rhesus monkeys fed a normal chow diet were made hypertriglyceridemic via administration of a high-fructose supplement for 16 weeks and then treated with ISIS 304801 (10, 20, and 40 mg/kg per week) for 12 weeks as the high-fructose diet was maintained. With fructose supplementation, plasma triglyceride levels were increased at least 3-fold over initial baseline in all treatment groups (0.49 mmol/L [43 mg/dL] at day 1 versus 1.6 mmol/L [142 mg/dL] at day 112, just before initiation of dosing). As was observed in the

Table 1. ApoC-III mRNA and Plasma Triglyceride Levels in Multiple Preclinical Models After Administration of Species-Specific ApoC-III Antisense Oligonucleotides

<table>
<thead>
<tr>
<th>Model/Diet</th>
<th>Hepatic ApoC-III mRNA (% Reduction)</th>
<th>Plasma TG, mmol/L</th>
<th>ApoC-III ASO</th>
<th>TG (% Change) vs Control ASO</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice (chow)†</td>
<td>−66</td>
<td>1.01±0.10</td>
<td>0.88±0.05</td>
<td>0.71±0.03†</td>
</tr>
<tr>
<td>C57BL/6 mice (Western diet)†</td>
<td>−90</td>
<td>1.02±0.06</td>
<td>1.14±0.06</td>
<td>0.64±0.04‡</td>
</tr>
<tr>
<td>Ldr/− mice (chow)†</td>
<td>−70</td>
<td>1.44±0.06</td>
<td>1.82±0.12</td>
<td>1.33±0.07†</td>
</tr>
<tr>
<td>Ldr/− mice (Western diet)†</td>
<td>−95</td>
<td>6.98±0.92</td>
<td>6.97±0.68</td>
<td>3.97±0.53‡</td>
</tr>
<tr>
<td>Ob/Ob mice (chow)†</td>
<td>−90</td>
<td>1.91±0.04</td>
<td>1.61±0.12</td>
<td>0.83±0.04†</td>
</tr>
<tr>
<td>CETP transgenic Ldr/− mice (Western diet)‡</td>
<td>−98</td>
<td>6.50±0.62</td>
<td>7.17±0.34</td>
<td>1.35±0.32‡</td>
</tr>
<tr>
<td>Sprague Dawley rats (fructose)§</td>
<td>−84</td>
<td>4.77±0.35</td>
<td>3.84±0.34</td>
<td>0.66±0.11†</td>
</tr>
<tr>
<td>ZDF rat (chow)§</td>
<td>−90</td>
<td>7.20±1.00</td>
<td>5.74±0.64</td>
<td>0.66±0.01†</td>
</tr>
</tbody>
</table>

ApoC-III indicates apolipoprotein C-III; ASO, antisense oligonucleotide; CETP, cholesteryl ester transfer protein; Ob/Ob mice, obese mice with a leptin deficiency; TG, triglyceride; and ZDF, Zucker diabetic fatty.

*Values represent mean±SEM. Plasma TG levels were evaluated after a 4-h fast.
†Denotes significantly different (P<0.05) from control ASO.
‡Denotes significantly different (P<0.05) from control ASO.
§Rats were administered 25 mg/kg per wk of ISIS 440726 for 6 wk.

ApoC-III protein and triglyceride were reduced in VLDL particles, and an increase in cholesteryl esters was observed at the highest ASO dose (Figure 4A). Except for the absence of apoC-III protein, LDL particle composition was unaffected (data not shown). We also evaluated the effects of the mouse-specific apoC-III ASO on apoB-containing lipoprotein particle size and demonstrated that there was a reduction in large VLDL and chylomicron particles and a modest reduction in small VLDL particles (Figure 4B).
normo-lipidemic monkeys described above in the pilot study, ISIS 304801 significantly reduced hepatic apoC-III mRNA and plasma apoC-III protein, while enhancing postprandial triglyceride clearance. C57BL/6 mice (n=5/group), fed either a chow or Western diet, were administered 12.5, 6.3, or 3.1 mg/kg per week of the murine apoC-III ASO for 6 weeks. A, Hepatic murine apoC-III expression was analyzed by quantitative polymerase chain reaction. B, Western blot analysis of apoC-III protein isolated from chow- and Western diet–fed mice. C, Postprandial triglyceride clearance was quantitated in the 12.5 mg/kg per week cohort by administering a fat bolus to chow-fed C57BL/6 fasted mice and measuring plasma triglyceride concentrations every hour for 4 hours. D, The mean area under the curve (AUC) was significantly reduced in apoC-III ASO-treated mice after the fat challenge. E, Postprandial TG absorption was quantitated in the 12.5 mg/kg per week cohort by first administering poloxamer 407 to chow-fed C57BL/6 fasted mice. One hour later, a 3H-triolein bolus was administered via oral gavage, and counts per minute was quantitated in plasma at 90 and 180 minutes after gavage. *Significant difference (P<0.05) from control ASO in chow-fed C56BL/6 mice; †Significant difference (P<0.05) from control ASO in Western diet–fed C57BL/6 mice.

Figure 2. Administration of the murine apolipoprotein C-III (apoC-III) antisense oligonucleotide (ASO; ISIS 440726) leads to dose-dependent reductions in hepatic apoC-III mRNA and plasma apoC-III protein, while enhancing postprandial triglyceride clearance.

Tolerability of Species-Specific ApoC-III ASOs in Preclinical Studies

In addition to specific pharmacological end points, we routinely monitor various factors, including plasma liver transaminases, and other metabolic parameters (ketones as well as liver, spleen, and body weights). In all preclinical rodent and nonhuman primate studies, there were no significant changes in plasma liver transaminases (Online Tables II and III), or other metabolic parameters when compared with controls, indicating that the apoC-III ASO was well tolerated (data not shown). Additionally, our data indicate that there were no statistically significant increases in hepatic triglyceride in mice or monkeys when compared with control animals (Figure 3C, Online Figure VD).
Pharmacodynamic Evaluations

Based on the preclinical efficacy data, a phase I double-blind, placebo-controlled, dose escalation clinical study (ISIS 304801-CS1) was performed to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of ISIS 304801 in healthy human volunteers (see online Data Supplement material for study details). Consistent with preclinical data, ISIS 304801 administered to healthy human volunteers produced dose-dependent and prolonged reductions in apoC-III with concomitant lowering of triglyceride levels (Figures 6A and B, Table 2). For example, median percentage reductions from baseline values in apoC-III of 19.7%, 17.3%, 70.5%, and >77.5% were observed in the 50-, 100-, 200-, and 400-mg multiple dose groups, respectively, 1 week after the last dose (day 29). Consistent with the changes in apoC-III, triglyceride levels were also reduced by 19.5%, 25.0%, 43.1%, and 43.8% in the same dosing cohorts. Despite the small sample size (n=3), the median percentage change from baseline at day 29 was the lowest possible at the 200- and 400-mg doses groups compared with placebo, and a correlation was observed between changes in triglyceride and apoC-III levels (r=0.899). Although the size of the study precludes a definitive conclusion about the effects of the 50 and 100 mg doses, the reductions on apoC-III and triglyceride were substantial. Reductions in apoC-III and triglyceride levels were sustained for at least 4 weeks after the last dose in the higher dose cohorts consistent with the long terminal elimination half-life of the drug.

Other lipid parameters were also evaluated (Table 2). As expected, LDL values did not change in this small study in normal subjects, whereas HDL levels tended to increase, although these changes were not dose dependent.

Pharmacokinetic Profile After Single and Multiple Dosing

Clinical pharmacokinetics were similar to those observed for other drugs of this chemical class.31 ISIS 304801 demonstrated dose-dependent increases in plasma exposure as measured by peak concentrations (Cmax) and AUC0-24. After a single subcutaneous injection (day 1), mean time to maximum plasma concentrations (Tmax) was between 2 and 4
hours, depending on the dose (Online Table IV). Exposure as measured by AUC_{0-24} also increased as a function of dose. The mean residence time in plasma was relatively short, consistent with the rapid distribution from the plasma into tissues. In the multiple dose cohorts (Online Table V), there was no plasma accumulation after multiple doses, with C_{max} and AUC values remaining similar after the first and last doses in each dose group. Plasma elimination half-life values ranged from 11.7 to 31.2 days, with clearance primarily a result of urinary excretion of metabolites.

Safety and Tolerability
ISIS 308401 was generally well tolerated. There were no serious adverse events and no early terminations of dosing because of an adverse event. The most common adverse event was mild injection site reaction, a typical response to subcutaneously administered drugs.52 No subject dosed with placebo complained of injection site reactions, although 13 of 25 (52%) subjects dosed with ISIS 304801 experienced at least one. Approximately 1 of 6 injections (median, 17%) led to an injection site reaction, the majority of which resolved within an hour. Single transient increases in C-reactive protein were reported in 7 of 25 (28%) subjects who received ISIS 304801. These increases were dose dependent, ranging from 7 to 29 mg/L, and had no associated symptoms. All other safety evaluations were clinically unremarkable across treatment groups.
Discussion

Because of the central role of apoC-III in triglyceride homeostasis and a compelling body of human genetic evidence linking elevated plasma apoC-III levels with hypertriglyceridemia, metabolic syndrome, and proinflammatory conditions, we developed species-specific antisense inhibitors to suppress apoC-III biosynthesis and evaluated their pharmacology in relevant rodent and nonhuman primate dyslipidemia and disease preclinical models and, more recently, in a phase I trial conducted in healthy human subjects. These studies demonstrated that apoC-III inhibition led to a variety of potential cardioprotective effects, including (1) significantly reduced plasma apoC-III and triglyceride, (2) enhanced postprandial triglyceride clearance, (3) favorable effects on VLDL particle composition, and (4) suggestions of enhanced HDL levels.

Several key observations that were essential for understanding the pharmacology of apoC-III inhibition are derived from rodent and monkey studies, including those performed in the well-characterized murine hypotriglyceridemic apoC3−/− model. For example, administration of the species-specific ASO to those mice did not affect their already low plasma triglyceride levels, indicating that the effects of the compound were apoC-III dependent. As described above, apoC-III ASOs also significantly enhanced postprandial triglyceride clearance in mice and nonhuman primates, data consistent with observations in the apoC3−/− mice, and in the Old World Amish possessing the R19X null mutation. Interestingly, postprandial clearance was more extensive in apoC3−/− mice, a whole body knockout of the target, compared with apoC-III ASO-treated mice, where suppression is more extensive in liver when compared with the proximal small intestine, where apoC-III plays an important role in chylomicron formation. These data suggest that intestinal apoC-III expression also plays an integral role in plasma triglyceride homeostasis.

Although the pharmacological outcome of inhibiting apoC-III on triglyceride and VLDL is predictable, the relationship to LDL levels is less clear. Some triglyceride-lowering agents, such as fish oils, have been shown to increase LDL in some instances. Although those agents were not evaluated in these studies, it is noteworthy that apoC-III ASO treatment alone did not modulate LDL levels in rodents, hypertriglyceridemic monkeys, and healthy volunteers in the phase I study. There is, however, considerable evidence that apoC-III present on

Figure 5. The human apolipoprotein C-III (apoC-III) antisense oligonucleotide, ISIS 304801, reduced plasma triglycerides, very low-density lipoprotein (VLDL)+chylomicron triglyceride, and decreased postprandial triglyceride excursion, while increasing high-density lipoprotein-cholesterol (HDL-C) in hypertriglyceridemic rhesus monkeys. Monkeys (n=5 per group) that were made hypertriglyceridemic with fructose supplementation were treated for a total weekly dose of 10 mg/kg per week ISIS 304801 or PBS over 12 weeks and plasma, A, total triglyceride, B, VLDL and chylomicron triglyceride, and C, HDL-C levels were quantified using nuclear magnetic resonance analysis. D, A postprandial triglyceride excursion test was performed in a separate cohort of rhesus monkeys (n=5 per group) that were treated with a total weekly dose of 10 mg/kg per week ISIS 304801 or PBS for 10 weeks. Postprandial mean plasma triglyceride concentrations (mmol/L)±SEM were measured after 0, 1, 2, 3, and 4 hours. Below each of the 4 histograms, a corresponding table provides the mean value for each data point plotted. Data are plotted as mean (mmol/L) change from baseline±SEM. *Significant difference from PBS cohort using 1-way ANOVA post hoc Tukey multicomparison test (P<0.05).
apoB-containing particles is proatherogenic. For example, in the Cholesterol and Recurrent Events trial, it was shown that in patients with coronary artery disease, increased apoC-III concentrations in VLDL+LDL were a significant predictor for a recurrent coronary event. Furthermore, increased serum apoC-III concentrations were associated with elevated levels of the small, dense LDL particles. Additionally, in patients with type 2 diabetes mellitus, apoC-III–enriched LDL particles had increased binding to arterial wall proteoglycans. Our data showing the depletion of apoC-III protein from VLDL in Western diet–fed Ldlr−/− mice suggest that antisense inhibition of apoC-III may produce less proinflammatory apoB-containing lipoprotein particles. Further studies in more hypertriglyceridemic states (>5.7 mmol/L [>500 mg/dL]) will be necessary to elucidate the effects of apoC-III inhibition in mixed dyslipidemia.

The well-established link between high plasma triglyceride and low HDL would suggest that the plasma triglyceride–lowering effects of apoC-III inhibition could lead to increases in HDL. Furthermore, there is evidence that apoC-III bound to HDL particles may produce a dysfunctional state with reduced cardioprotective properties. In preclinical models possessing CETP, that is, CETP transgenic Ldlr−/− mice, nonhuman primates, as well as in the phase I clinical trial, apoC-III inhibition led to either significant increases (Online Table VII) or elevation trends (nonhuman primate study and phase I study) in HDL levels. These effects are, once again, consistent with observations

**Table 2. Dose-Dependent Reduction in ApoC-III and Triglyceride Levels in Healthy Human Volunteers After Short-Term Multiple Dose Treatment With ISIS 304801**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Median Age (%) Change From Baseline 1 wk After Last Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoC-III</td>
<td>-11.0 50 mg (n=3) -19.7 100 mg (n=3) -17.3 200 mg (n=3) -70.5* 400 mg (n=3) &gt;-77.5*</td>
</tr>
<tr>
<td>TG</td>
<td>28.5 50 mg (n=3) -19.5 100 mg (n=3) -25.0 200 mg (n=3) -43.1 400 mg (n=3) -43.8</td>
</tr>
<tr>
<td>HDL-C</td>
<td>2.1 50 mg (n=3) 19.0 100 mg (n=3) 0.0 200 mg (n=3) 13.9 400 mg (n=3) 8.0</td>
</tr>
<tr>
<td>LDL-C</td>
<td>5.5 50 mg (n=3) 18.4 100 mg (n=3) -3.6 200 mg (n=3) -3.2 400 mg (n=3) -3.9</td>
</tr>
</tbody>
</table>

ApoC-III indicates apolipoprotein C-III; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and TG, triglyceride.

Values are the median percentage change from baseline, 1 wk after the last dose of study drug (day 29). The percentage change from baseline for each dose group was compared with pooled placebo using the exact Wilcoxon Rank Sum test.

*Statistic test result (P=0.0571) was the lowest P value possible for the sample size.
in the Old Order Amish. It is important to note that both the nonhuman primates and subjects in our clinical trial had normal to modestly elevated triglyceride levels, raising the possibility that the HDL effects of apoC-III inhibition could be more robust in a hypertriglyceridemic state. Furthermore, as HDL increases were not seen in CETP-deficient models, this may also suggest that the HDL raising properties of apoC-III are CETP dependent. Whether these effects are attributable to changes in CETP substrate availability or direct effects on CETP enzymatic activity are currently under investigation.

In all of the preclinical studies, apoC-III ASOs were well tolerated. In rodent models, there was no change in body weights (data not shown) or plasma transaminases with any apoC-III ASO dose. Although previous studies have indicated that high-fat fed apoC3−/− mice developed hepatic steatosis, we did not observe an increase in liver triglyceride in mice or nonhuman primates. The fact that ASO administration does not affect triglyceride secretion is consistent with these observations.

The human apoC-III antisense drug, ISIS 304801, was well tolerated in healthy human volunteers, with no unexpected safety signals identified across the full dose range tested. The pharmacokinetic and pharmacodynamic profiles were predictable based on the preclinical results and previous preclinical and clinical experience with other ASO drugs in its class. A profound dose-dependent reduction of apoC-III up to 90% from baseline occurred with an associated dose-dependent reduction in triglyceride up to 80% in subjects administered active drug for 4 weeks. This pharmacological response was durable, with median apoC-III levels remaining below baseline for at least 1 month at the higher doses of 200 and 400 mg.

In conclusion, ASO-mediated suppression of apoC-III has demonstrated robust triglyceride and VLDL lowering across multiple rodent models, nonhuman primates, and man. In addition, in hypertriglyceridemic monkeys and healthy human subjects, apoC-III inhibition seemed to raise HDL levels with no adverse effects on LDL. Based on the favorable preclinical and clinical response was durable, with median apoC-III levels remaining below baseline for at least 1 month at the higher doses of 200 and 400 mg.

In conclusion, ASO-mediated suppression of apoC-III has demonstrated robust triglyceride and VLDL lowering across multiple rodent models, nonhuman primates, and man. In addition, in hypertriglyceridemic monkeys and healthy human subjects, apoC-III inhibition seemed to raise HDL levels with no adverse effects on LDL.

Acknowledgments
We thank Drs Brett P. Monia and Frank C. Bennett for their thoughtful review of this article. In addition, we also acknowledge Tracy Reigle for her valuable help in formatting all of the figures and tables.

References


**What Is Known?**
- Elevated triglycerides are an independent risk factor for cardiovascular disease, and very high triglyceride levels (>5.7 mmol/L) are associated with an enhanced risk of pancreatitis.
- Current therapeutic agents for the treatment of very high triglyceride levels are limited.
- Apolipoprotein C-III (apoC-III) is synthesized principally in the liver, and it regulates serum triglyceride levels.
- Loss-of-function variants of apoC-III in a group of Old Order Amish are associated with lower serum triglyceride levels and are cardioprotective.

**What New Information Does This Article Contribute?**
- ApoC-III biosynthesis could be selectively inhibited by antisense oligonucleotides.
- ApoC-III antisense oligonucleotide treatment produces consistent and significant reductions in serum apoC-III and triglyceride levels in rodents, nonhuman primates, and man.
- ApoC-III antisense oligonucleotide drugs are well tolerated in preclinical models and in a clinical setting, with no evidence of hepatotoxicity.

**Novelty and Significance**
Individuals with very high triglyceride (>5.7 mmol/L) and apoC-III levels are at increased risk for developing cardiovascular disease, metabolic syndrome, diabetes mellitus, and pancreatitis. Although several therapeutic agents affect triglyceride levels, there is an unmet need for more effective therapies. Given the importance of apoC-III in the regulation of triglyceride homeostasis, we developed antisense inhibitors to demonstrate that reduction of apoC-III could produce therapeutic benefit. In preclinical rodent and nonhuman primate models and, most importantly, in man, we demonstrate that selective inhibition of apoC-III is well tolerated, and that it results in significant, prolonged reductions in apoC-III and triglyceride levels, enhanced postprandial triglyceride clearance. The human apoC-III antisense drug may be used as a monotherapy or in combination with other agents, for example, fibrates. Drug–drug interactions are not anticipated because antisense oligonucleotides are metabolized by nucleases rather than by metabolic pathways used by traditional small molecules, such as the cytochrome P450 system. These findings support clinical evaluation of this apoC-III drug in subjects with severely elevated triglyceride levels and type 2 diabetics with moderately elevated triglyceride levels and uncontrolled glucose levels.
Antisense Oligonucleotide Inhibition of Apolipoprotein C-III Reduces Plasma Triglycerides in Rodents, Nonhuman Primates, and Humans


Circ Res. 2013;112:1479-1490; originally published online March 29, 2013; doi: 10.1161/CIRCRESAHA.111.300367

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Material

Therapeutic Inhibition of Apolipoprotein C-III Reduces Plasma Triglycerides in Rodents, Nonhuman Primates and Man


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Supplemental Detailed Material and Methods

Oligonucleotides

The species-specific apoC-III sequences evaluated were as follows: murine ASO-ISIS 440726 (5'-CCAGCTTTATAGGACAGC-3'), rat ASO-ISIS 353982 (5'-GAGAATATACTTTCCCTTA-3'), human ASO-ISIS 304801 (5'-AGCTTCTTGTCCAGCTTTAT-3'), and control ASO-ISIS 141923 (5'-CCTTCCCTGAAGGTTCCCTCC-3'), with underlining indicating 2' MOE modified bases.

Preclinical Pharmacology

Humane Care of Animals

Treatment of the mice, rats (Isis Pharmaceuticals) and monkeys (Korea Institute of Toxicology) was in accordance with the conditions specified in the Guide for the Care and Use of Laboratory Animals (ILAR publication, 1996, National Academy Press). The study protocols were approved by each testing facilities IACUC prior to dose administration.

Murine Studies

Six week old male C57BL/6 mice, Ldlr +/- mice (B6.129S7-Ldlr<sup>tm1Her</sup>/J, Jackson Laboratories, Bar Harbor, ME), Ob/Ob (B6.Cg-Lep<sup>ob</sup>/J) and apoC-III +/- mice (B6.129P2-Apoc3<sup>tm1Unc</sup>), were housed 4-5 to a cage on a 12 hr light-dark cycle for the duration of the study. Ldlr +/- mice were used because they 1) possess a lipoprotein cholesterol distribution similar to humans and 2) with a dietary challenge they develop marked hypertriglyceridemia. Ob/Ob mice were used because they are a well characterized diabetic model of hypertriglyceridemia. Mice were fed a standard chow diet or a Western diet (Harland Teklad Diet 88137, Harland, Indianapolis, IN) consisting of 42% of calories as fat and 0.15% cholesterol for 1 week. The mice were then bled, randomized based on total plasma cholesterol and plasma TG levels, and administered either saline,
control ASO (12.5 mg/kg/wk), or an apoC-III ASO at three different doses (12.5, 6.3, or 3.1 mg/kg/wk) for a period of six weeks while remaining on diet. After six weeks of ASO treatment, mice were fasted for 4 hours, then sacrificed, and plasma and tissues were collected for further detailed analysis.

**CETP Transgenic, Ldlr -/- Mice**

The human (hu) CETP transgenic (Tg) mice used in these studies were a gift from the lab of Linda Curtiss and the creation of these mice has been described in detail\(^1\). Due to the inclusion of the CETP protein, these mice provided a more accurate model of apoC-III’s triglyceride lowering effects on HDL metabolism. These mice were generated by breeding the huCETP Tg animals with mice lacking a functional LDL receptor. The heterozygous progeny were backcrossed resulting in homozygous huCETP Tg, Ldlr -/- mice. Mice were switched from chow to a Western diet (Harland Teklad Diet 88137, Harland, Indianapolis, IN) consisting of 42% of calories as fat and 0.15% cholesterol one week before baseline plasma samples were drawn by a retro-orbital bleed. Mice in the treatment groups were balanced according to baseline plasma lipids and body weight. The treatment groups were then administered either saline, a control ASO (12.5 mg/kg/wk), or the human apoC-III ASO (12.5 mg/kg/wk) for 4 weeks.

**Rats**

**Fructose-fed**

Six week old male Sprague Dawley rats were housed two to a cage on a 12 hr light-dark cycle for the duration of the study. Rats were randomized based on total plasma cholesterol and plasma TG levels (n=6/group) and then fed a high fructose diet (Tekland Harland TD.89247, Harland, Indianapolis, IN). Upon initiation of high fructose diet feeding, rats were administered either saline, a control ASO (12.5 mg/kg/wk), or the rat apoC-III ASO (6.3, 12.5, or 25 mg/kg/wk). All apoC-III ASO doses were administered
as weekly subcutaneous injections while. After six weeks of drug treatment, rats were fasted for four hours, then sacrificed and plasma and liver tissue collected for analysis.

**Zucker Diabetic Fatty Rats**

Male Zucker diabetic fatty rats (ZDF-Lepr<sup>fa</sup>/Crl), approximately 6-7 weeks of age, were housed two per cage and fed a standard chow diet. Rats were randomized based on fasting plasma TG (n=8/group) and then administered, via subcutaneous injection, either saline, control ASO (25 mg/kg/wk), or the rat apoC-III (25 mg/kg/wk) for a period of six weeks. Two days following the final injection, rats were fasted for four hours, then sacrificed and plasma and tissues were collected for analysis.

**Hypertriglyceridemic Rhesus Monkeys**

A 12 week study in nonhuman primates was performed by the Korea Institute of Toxicology (P.O. Box 123, 100 Jang-dong, Yuseong-gu, Daejeon 305-600, Korea) under the supervision of Hong-Soo Lee, DVM, Study Director. Male Rhesus monkeys (*Macaca mulatta*, Guangxi Grandforest Scientific Primate Co, LTD, Guangxi, China) were made hypertriglyceridemic via administration of a high fructose supplement (i.e. Kool Aid®, which contains ~15% fructose) that was supplied in the morning for 16 weeks prior to group randomization. Animals received the Kool Aid® mixture throughout the study. To assure sufficient TG level elevations, blood samples for serum chemistry were collected from all animals at 1 and 2 weeks prior to randomization. Monkeys were assigned to 4 treatment groups (n=5/group) and administered either PBS, or 10, 20, and 40 mg/kg/wk of ISIS 304801 (5, 10 20 mg/kg twice weekly SQ injection) for 13 weeks after an initial loading regimen consisting of every other day administration for the first week. Animals were sacrificed on Day 86 approximately 48 hrs after the last dosing on Days 84 or 85.

**Postprandial TG Excursion Test in HTG Rhesus Monkeys**

Rhesus monkeys were maintained on a high fructose supplement and were fed about 60 g of primate diet (Certified Primate Diet #5048, PMI Nutrition International, Inc.,
USA) provided to each monkey in all groups twice daily. Small bits of fruit, cereal or other treats were occasionally given to the monkeys as part of the Testing Facility environmental enrichment program. After 10 week ASO administration, were fasted for 8 hours and blood samples were drawn just prior to (time = 0 hour) and 1, 2, 4, and 6 hours after ingestion of the primate diet to assess post-prandial TG levels. The monkeys were rested and remained otherwise fasting during the 6 hours post-meal challenge. Blood samples (approximately 5 mL) were put into tubes containing EDTA-2K and the tubes were centrifuged (approximately 3000 rpm, 10 minutes, at room temperature) to obtain plasma samples for TG analysis.

**RNA Purification and ApoC-III Expression Analysis (RT-PCR)**

RNA was purified and subjected to RT-PCR analysis as described previously\(^3\). The Perkin-Elmer ABI Prism 7700 Sequence Detection System, which uses real-time fluorescence RT-PCR detection, was used to quantify mRNA. RNA transcripts were normalized to total RNA levels using cyclophilin A. Sequences of primer probe sets are available upon request.

**Plasma Chemistry Analyses**

For preclinical studies, plasma aminotransferases (AST, ALT), total plasma cholesterol (TPC), plasma TG, non-HDL-C, HDL-C, glucose, and non-esterified fatty acids (NEFA) were quantitated using an Olympus clinical analyzer as described previously\(^4\).

**ApoC-III Plasma Protein Analysis (Turbidometric Assay)**

Approximately 100 µl of plasma isolated from mice and monkeys was analyzed without dilution using an Olympus Clinical Analyzer and a commercially available turbidometric apoC-III assay (Kamiya, Cat# KAI-006, Kamiya Biomedical, Seattle, WA). The assay protocol was performed as described by the vendor.

**Western Blotting of ApoC-III**
Diluted serum or 5 µg of isolated VLDL protein was run on a 5-20% tris-glycine gel, and immunoblotted using apoC-III antibodies raised in a rabbit to a peptide consisting of aa 86-99 of the murine apoCIII protein (Genscript, Piscataway, NJ). The blots were then washed, incubated with a 800 nm fluorescent tagged anti-rabbit secondary antibody (Licor, Lincoln, NB), and visualized in an Odyssey imaging device (Licor, Lincoln, NB).

**Compositional Analysis of ApoB-containing Lipoproteins**

Sequential density ultracentrifugation was carried out to isolate VLDL (d<1.006) and LDL (1.019<d<1.063) from plasma from western diet fed Ldlr -/- mice treated with ASO for six weeks. Colorimetric assays were used to quantitate free cholesterol (FC), total cholesterol (TC), phospholipid (Wako Chemicals, Richmond, CA), and TG (Roche Diagnostics, Indianapolis, IN). CE mass was calculated by subtracting FC from TC and multiplying by 1.67 (the average mass of the FA component of the CE molecule). Total protein was quantitated using protein analysis kit (BioRad Laboratories, Hercules, CA).

**Lipoprotein Particle Analysis**

Plasma samples drawn on Day -7, 1, 16, 30 and 86 (monkey study) or after 6 weeks of apoC-III ASO treatment (mice) were selected for NMR lipoprotein particle analysis, which was performed by LipoScience™ (Raleigh, NC) using previously described methods⁵.

**Triglyceride Clearance**

After five weeks of ASO treatment, mice underwent fat clearance tests as described previously⁶. Briefly, mice were fasted overnight, orally gavaged with 300 µl of olive oil, and retro-orbital bleeds collected at the indicated time points. The TG concentration of each time point was then quantitated using a colorimetric assay (Wako Chemicals, Richmond, CA).
**Intestinal Fat Absorption**

Intestinal fat absorption was quantitated as described previously\(^7\). Briefly, chow fed mice administered 12.5 mg/kg/wk of either control or apoC-III ASO for 6 weeks were fasted overnight and then received an intraperitoneal injection of poloxamer 407 at a dose of 1 mg/g body weight. One hour after injection, a baseline retro-orbital bleed was collected and the mice were orally gavaged with 2.5 µCi of \(^3\)H-triolein in 200 µl of olive oil. Animals were then bled 90 and 180 min post gavage, and relative counts per minute (CPM) values were quantified from 20 µl of serum by liquid scintillation counting (Beckman® LSC Instrument).

**Quantitation of Liver TG Secretion**

To quantitate liver TG secretion, mice were administered poloxamer 407, a nonionic detergent that has been shown to inhibit VLDL catabolism without causing some of the unwanted side effects associated with Triton WR-1339 administration, as described previously\(^8\).

**Liver Lipids**

Liver TG concentrations were quantitated in mice and monkeys as described previously\(^9\).

**Statistical Analyses**

For preclinical rodent and rhesus monkey studies, statistical significance (\(p<0.05\)) was determined by 1 way ANOVA with Tukey’s post hoc analysis using GraphPad Prism 5 software.

**Phase I Clinical Trial with ISIS 304801**
The Phase I clinical trial was conducted by a contract research organization, Kendle Early Stage (Toronto, Canada). Thirty-three human subjects ranging in age from 18 to 55 years, with a body mass index (BMI) less than 32 kg/m², were enrolled. Prior to enrollment, subjects submitted to pretrial screening. Pregnant women, nursing mothers and women of child bearing potential were excluded, as were subjects that displayed abnormal ECGs or laboratory tests, or were receiving prescription medications. Anyone who smoked >10 cigarettes a day or were alcohol and/or substance abusers were excluded. All subjects gave their informed consent prior to enrollment.

Subjects randomized to the initial 4 sequential dose cohorts were administered a single subcutaneous (SC) injection of placebo, 50, 100, 200 or 400 mg ISIS 308401. Subsequent multi-dose cohorts received three doses of placebo or ISIS 308401 (50, 100, 200, or 400 mg) in the first week (loading schedule- Days 1, 3 and 5) followed by three weekly doses (Days 8, 15, 22). During the first week, subjects were housed in the study center where they were administered drug and evaluated on an out-patient basis. During this time, the subjects were clinically evaluated daily and subjected to laboratory evaluations prior to every dose. Clinical and laboratory evaluations were also performed prior to the next three weekly doses. All subjects were followed for a minimum of 50 days post the initial dose.

**Safety Assessment**

Safety and tolerability were assessed by the incidence, severity and dose relationship of adverse events (AEs) and changes in laboratory evaluations. Laboratory evaluations included routine hematology with differential, blood chemistry, coagulation parameters and urinalysis.

**Lipid and Lipoprotein Analyses**

Fasting blood samples were analyzed for total lipids and lipoproteins using an Olympus clinical analyzer (MEDPACE reference laboratory, Cincinnati OH). ApoC-III
was evaluated using an immuno-turbidimetric assay on a Daytona clinical analyzer (Daytona, Ramsey, MN). In some cases plasma apoC-III concentrations were reduced below the level of detection (<1mg/dL) after treatment with ISIS 304801. To determine significance, such samples were given a value of 1mg/dL prior to statistical evaluation.

**Pharmacokinetic Analyses**

Plasma pharmacokinetic parameters were determined by non-compartmental methods using plasma concentration-time data collected after single and multiple dose\textsuperscript{10}. Plasma concentrations were measured at PPD Development (Richmond, VA) using a validated hybridization-ELISA assay with a 0.5 to 100 ng/mL quantitation range. Terminal elimination half-life values were estimated from 7 days to 33 days after the final dose in each subject from the multiple dose cohorts. Pre-dose/trough concentrations were obtained on days 1, 15, 22 and 29.

**Statistical Analysis**

All human subjects who received at least one dose of ISIS 304801 or placebo were included in the safety analyses. Placebo subjects were pooled and analyzed as a group. Baseline was defined as the observation before the first dose for each subject. Lipid and lipoprotein parameters were analyzed on the per-protocol population. Descriptive statistics for apoC-III and TG are presented by dose over time. Percent change from baseline for each ISIS 304801 dose group was compared to the pooled placebo using the exact Wilcoxon rank sum test. While statistical tests were performed on all lipid parameters measured, only the significant changes are highlighted in the results section.


Online Table I. Baseline Characteristics of Healthy Human Volunteers Assigned to the Multi-Dose Cohorts

<table>
<thead>
<tr>
<th>Median</th>
<th>Placebo (n=4)</th>
<th>50 mg (n=3)</th>
<th>100 mg (n=3)</th>
<th>200 mg (n=3)</th>
<th>400 mg (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M:F)</td>
<td>3:1</td>
<td>3:0</td>
<td>3:0</td>
<td>3:0</td>
<td>3:0</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>43</td>
<td>40</td>
<td>40</td>
<td>43</td>
<td>40</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.7</td>
<td>24.0</td>
<td>27.3</td>
<td>28.0</td>
<td>27.5</td>
</tr>
<tr>
<td>Lipids &amp; Lipoproteins (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoC-III *</td>
<td>6.3</td>
<td>10.4</td>
<td>9.5</td>
<td>11.6</td>
<td>8.7</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.1</td>
<td>1.4</td>
<td>1.1</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>5.0</td>
<td>4.1</td>
<td>5.1</td>
<td>4.8</td>
<td>4.7</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.2</td>
<td>1.1</td>
<td>1.2</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Non-HDL-C</td>
<td>3.5</td>
<td>3.1</td>
<td>3.9</td>
<td>3.9</td>
<td>3.3</td>
</tr>
<tr>
<td>LDL-C</td>
<td>2.9</td>
<td>2.4</td>
<td>3.4</td>
<td>2.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Table presents results from analysis of the per-protocol population.

* Presented in mg/dL
Online Table II. Effect of ApoC-III ASO Treatment on Plasma ALT, Total Cholesterol, TG, Non-HDL-C, HDL-C, Glucose and Non-Esterified Fatty Acid Levels in C57BL/6 Mice on Chow & Western Diet After 6 Weeks of Treatment

<table>
<thead>
<tr>
<th>Diet</th>
<th>Treatment (mg/kg/wk)</th>
<th>ALT (U/L)</th>
<th>Total Cholesterol (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>Non-HDL-C (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>Glucose (mmol/L)</th>
<th>Non-Esterified Fatty Acid (mEq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>Saline</td>
<td>22.8 ± 2.0</td>
<td>2.25 ± 0.10</td>
<td>1.01 ± 0.10</td>
<td>0.45 ± 0.02</td>
<td>1.94 ± 0.09</td>
<td>13.44 ± 0.40</td>
<td>0.91 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Control ASO (12.5)</td>
<td>41.0 ± 3.7</td>
<td>2.53 ± 0.15</td>
<td>0.88 ± 0.05</td>
<td>0.60 ± 0.03</td>
<td>2.04 ± 0.13</td>
<td>11.89 ± 0.99</td>
<td>0.73 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>ApoC-III ASO (12.5)</td>
<td>35.3 ± 3.0</td>
<td>2.29 ± 0.10</td>
<td>0.71 ± 0.03</td>
<td>0.45 ± 0.05</td>
<td>1.98 ± 0.07</td>
<td>12.21 ± 0.34</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>ApoC-III ASO (6.3)</td>
<td>27.2 ± 4.9</td>
<td>2.18 ± 0.13</td>
<td>0.79 ± 0.06</td>
<td>0.40 ± 0.03</td>
<td>1.89 ± 0.12</td>
<td>12.01 ± 12.38</td>
<td>0.74 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>ApoC-III ASO (3.1)</td>
<td>23.6 ± 2.8*</td>
<td>2.31 ± 0.06</td>
<td>0.86 ± 0.05</td>
<td>0.43 ± 0.01</td>
<td>1.98 ± 0.08</td>
<td>12.38 ± 0.62</td>
<td>0.71 ± 0.07</td>
</tr>
<tr>
<td>Western Diet</td>
<td>Saline</td>
<td>61.9 ± 14.9</td>
<td>6.28 ± 0.19</td>
<td>1.02 ± 0.06</td>
<td>1.41 ± 0.05</td>
<td>5.64 ± 0.16</td>
<td>21.26 ± 0.81</td>
<td>1.90 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Control ASO (12.5)</td>
<td>80.6 ± 25.5</td>
<td>5.3 ± 0.19</td>
<td>1.14 ± 0.06</td>
<td>1.20 ± 0.04</td>
<td>4.44 ± 0.22</td>
<td>20.08 ± 1.33</td>
<td>1.98 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>ApoC-III ASO (12.5)</td>
<td>33.6 ± 8.8</td>
<td>4.04 ± 0.26*</td>
<td>0.64 ± 0.04</td>
<td>0.78 ± 0.04*</td>
<td>3.91 ± 0.28</td>
<td>17.42 ± 1.10</td>
<td>1.47 ± 0.09*</td>
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<td></td>
<td>ApoC-III ASO (6.3)</td>
<td>91.0 ± 25.5</td>
<td>4.80 ± 0.26</td>
<td>0.57 ± 0.03</td>
<td>0.95 ± 0.07*</td>
<td>4.63 ± 0.17</td>
<td>19.86 ± 1.70</td>
<td>1.68 ± 0.07*</td>
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<tr>
<td></td>
<td>ApoC-III ASO (3.1)</td>
<td>38.2 ± 10.7</td>
<td>4.51 ± 0.14</td>
<td>0.72 ± 0.06</td>
<td>1.00 ± 0.02*</td>
<td>4.14 ± 0.16</td>
<td>18.36 ± 0.52</td>
<td>1.53 ± 0.05*</td>
</tr>
</tbody>
</table>

*Significant difference from control ASO (p<0.05)
### Online Table III. Effect of ApoC-III ASO Treatment on Plasma ALT, Total Cholesterol, TG, Non-HDL-C, HDL-C, Glucose and Non-Esterified Fatty Acid Levels in *Ldl-r -/-* Mice on Chow & Western Diet After 6 Weeks of Treatment

<table>
<thead>
<tr>
<th>Diet</th>
<th>Treatment (mg/kg/wk)</th>
<th>ALT (U/L)</th>
<th>Total Cholesterol (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>Non-HDL-C (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>Glucose (mmol/L)</th>
<th>Non-Esterified Fatty Acid (mEq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow Saline</td>
<td>97.0 ± 5.7</td>
<td>5.81 ± 0.27</td>
<td>1.44 ± 0.06</td>
<td>2.97 ± 0.16</td>
<td>2.97 ± 0.12</td>
<td>12.49 ± 0.75</td>
<td>0.83 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Control ASO (12.5)</td>
<td>77.1 ± 14.3</td>
<td>7.22 ± 0.44</td>
<td>1.82 ± 0.12</td>
<td>4.22 ± 0.43</td>
<td>2.97 ± 0.10</td>
<td>11.47 ± 0.52</td>
<td>0.80 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>ApoC-III ASO (12.5)</td>
<td>70.8 ± 11.2</td>
<td>6.20 ± 0.26</td>
<td>1.33 ± 0.07</td>
<td>3.36 ± 0.17</td>
<td>2.96 ± 0.09</td>
<td>12.43 ± 0.49</td>
<td>0.75 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>ApoC-III ASO (6.3)</td>
<td>111.1 ± 55.4</td>
<td>6.12 ± 0.24</td>
<td>1.42 ± 0.06</td>
<td>3.18 ± 0.14</td>
<td>2.97 ± 0.10</td>
<td>12.61 ± 0.28</td>
<td>0.61 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>ApoC-III ASO (3.1)</td>
<td>65.4 ± 9.3</td>
<td>6.52 ± 0.22</td>
<td>1.48 ± 0.04</td>
<td>3.50 ± 0.16</td>
<td>3.04 ± 0.06</td>
<td>13.10 ± 0.68</td>
<td>0.69 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Western Diet Saline</td>
<td>98.8 ± 20.3</td>
<td>38.43 ± 3.87</td>
<td>6.98 ± 0.92</td>
<td>26.29 ± 2.12</td>
<td>8.92 ± 0.70</td>
<td>13.34 ± 0.63</td>
<td>0.76 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Control ASO (12.5)</td>
<td>93.7 ± 15.8</td>
<td>41.34 ± 1.91</td>
<td>6.97 ± 0.68</td>
<td>28.96 ± 1.18</td>
<td>9.06 ± 0.63</td>
<td>14.26 ± 0.66</td>
<td>0.99 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>ApoC-III ASO (12.5)</td>
<td>86.4 ± 12.2</td>
<td>32.73 ± 3.46</td>
<td>3.97 ± 0.53</td>
<td>24.25 ± 2.34</td>
<td>7.39 ± 0.89</td>
<td>13.94 ± 0.85</td>
<td>0.79 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>ApoC-III ASO (6.3)</td>
<td>76.8 ± 9.6</td>
<td>30.84 ± 3.52</td>
<td>4.99 ± 0.69</td>
<td>22.92 ± 2.5</td>
<td>6.73 ± 0.84</td>
<td>13.42 ± 1.20</td>
<td>0.93 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>ApoC-III ASO (3.1)</td>
<td>68.0 ± 11.2</td>
<td>32.61 ± 2.27</td>
<td>4.85 ± 0.26</td>
<td>24.62 ± 1.52</td>
<td>7.10 ± 0.38</td>
<td>14.28 ± 1.05</td>
<td>1.07 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference from control ASO (p<0.05)
Online Table IV. Pharmacokinetic Summary: ISIS 304801-CS1 Single Dose

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th><strong>AUC</strong>&lt;sub&gt;0-24&lt;/sub&gt; (µg•hr/mL)</th>
<th><strong>C&lt;sub&gt;max&lt;/sub&gt;</strong> (µg/mL)</th>
<th><strong>T&lt;sub&gt;max&lt;/sub&gt;</strong> (hr)</th>
<th><strong>MRT&lt;sub&gt;last&lt;/sub&gt;</strong>&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>9.01 ± 3.74</td>
<td>1.12 ± 0.548</td>
<td>2 (2-4)</td>
<td>6.43 ± 0.925</td>
</tr>
<tr>
<td>100</td>
<td>22.2 ± 5.32</td>
<td>2.19 ± 0.727</td>
<td>3 (3-4)</td>
<td>6.95 ± 1.11</td>
</tr>
<tr>
<td>200</td>
<td>34.6 ± 15.1</td>
<td>2.84 ± 1.29</td>
<td>4 (3-6)</td>
<td>8.12 ± 0.987</td>
</tr>
<tr>
<td>400</td>
<td>143 ± 34.2</td>
<td>13 ± 2.35</td>
<td>3 (2-6)</td>
<td>7.63 ± 0.0987</td>
</tr>
</tbody>
</table>

*Mean Residence Time (MRT<sub>last</sub>) is 0 to 24 hours

Values are reported as means ± standard deviation, with the exception of **T<sub>max</sub>**, which is reported as median (min – max)
<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Profile</th>
<th>N</th>
<th>AUC$_{0-24}$ (µg•hr/mL)</th>
<th>C$_{max}$ (µg/mL)</th>
<th>T$_{max}$ (hr)</th>
<th>T$_{1/2λz}$(hr)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>First</td>
<td>3</td>
<td>4.11 ± 3.67</td>
<td>0.659 ± 0.368</td>
<td>2 (1.5-2)</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>Last</td>
<td>3</td>
<td>8.35 ± 2.57</td>
<td>0.885 ± 0.236</td>
<td>3 (3-8)</td>
<td>749 ± 253</td>
</tr>
<tr>
<td>100</td>
<td>First</td>
<td>3</td>
<td>20.9 ± 5.53</td>
<td>2.05 ± 0.979</td>
<td>4 (3-6)</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>Last</td>
<td>3</td>
<td>24.5 ± 9.23</td>
<td>2.9 ± 1.43</td>
<td>3 (3-4)</td>
<td>437 ± 259</td>
</tr>
<tr>
<td>200</td>
<td>First</td>
<td>3</td>
<td>49.1 ± 1.68</td>
<td>4.01 ± 0.814</td>
<td>2 (1.5-3)</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>Last</td>
<td>3</td>
<td>50.7 ± 6.48</td>
<td>4.12 ± 0.720</td>
<td>3 (3-4)</td>
<td>281 ± 77.2</td>
</tr>
<tr>
<td>400</td>
<td>First</td>
<td>4</td>
<td>128 ± 40.3</td>
<td>10.2 ± 3.06</td>
<td>3.5 (1.5-4)</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>Last</td>
<td>3</td>
<td>122 ± 39.8</td>
<td>9.56 ± 3.52</td>
<td>4 (3-6)</td>
<td>333 ± 121</td>
</tr>
</tbody>
</table>

Values are reported as means ± standard deviation, with the exception of Maximum Concentration (C$_{max}$), Maximum Plasma Concentration (T$_{max}$), Plasma Terminal Elimination Half-Life (T$_{1/2λz}$), which is reported as median (min – max)
Online Table VI. Baseline plasma TG, VLDL TG and HDL Levels in hypertriglyceridemic rhesus monkeys.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TG (mmol/L)</th>
<th>VLDL + Chylo TG (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (n=5)</td>
<td>1.19±0.19</td>
<td>0.76±0.18</td>
<td>2.36±0.16</td>
</tr>
<tr>
<td>10mg/kg/wk (n=5)</td>
<td>1.31±0.24</td>
<td>0.89±0.22</td>
<td>2.22±0.19</td>
</tr>
</tbody>
</table>

Concentration of plasma TG, VLDL TG and HDL-C (mmol/L±SEM) measured in unfasted PBS and ISIS 304801 10mg/kg/wk treatment groups after 16 weeks of high fructose diet and just prior to dose initiation.
Online Table VII. HDL-C levels in homozygous CETP Tg Ldlr -/- mice after 6 week ASO administration.

<table>
<thead>
<tr>
<th>CETP Tg Ldlr -/- Mice (Western Diet)*</th>
<th>HDL-C (mmol/L)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n=12)</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>Control ASO (n=14)</td>
<td>0.36±0.03</td>
</tr>
<tr>
<td>ApoC-III ASO (n=10)</td>
<td>1.76±0.24§</td>
</tr>
</tbody>
</table>

Homozygous CETP Tg Ldlr -/- mice were fed a Western Diet (Harland Teklad Diet 88137) and administered either saline (n=12), a control ASO (n=14) [12.5 mg/kg/wk], or an apoC-III ASO (n=10) [12.5 mg/kg/wk] for 6 weeks. Plasma HDL-C was quantified using the HDL Cholesterol E kit from Wako Chemicals.

‡Values represent mean±SEM.

§Denotes significantly different (P<0.05) from saline and control ASO treated mice.
**Online Figure I.**

**A**

![Diagram A](image)

*Housed in-clinic Day -1 to 2.*

**B**

![Diagram B](image)

**Single and multiple dose cohort schedules (ISIS 304801).** Study schedules for **A**, single and **B**, multi-dose cohorts. Healthy volunteers between 18 and 55 were randomized 3:1 active to placebo for each sequential dose cohort (50, 100, 200 and 400 mg). Outpatient visits were on days 4, 8 and 15 for single dose cohorts, and days 8, 9, 15, 16, 29, 36 and 50 for the multi-dose cohorts. Arrows denote study days that subjects were dosed. *Housed in-clinic Day -1 to 6, Day 22 to 23.*
Online Figure II.

Flow of study participants. * Investigator decision for withdrawal, based upon poor participant compliance with protocol schedule. Subject received 3 of 6 400-mg doses prior to discontinuation.
Online Figure III.

Map of human ASO hybridization sites tested for apoC-III mRNA suppression in HepG2 cells. At the top of the figure, the multiple 20mer ASO binding sites evaluated in HepG2 cells is illustrated. Immediately below the ASO map, the target subsequence (TSS) indicated in the first field identifies the GenBank NM_000040.1 sequence which was used to design all the complementary 20mer antisense sequences. The second field reveals the coding description sequence (CDS) and provides information on the relative positions of the 5' non translated region (UTR), coding region (CDR) and 3' UTR within the human apoC-III transcript, respectively. The third field provides the relative mapping postions of exons 1-4 within the human apoC-III gene. The fourth field provides the location of the signaling peptide and mature protein domains within the translated apoC-III protein sequence.
Online Figure IV.

Administration of the murine apoC-III ASO to chow fed C57BL/6 mice inhibits intestinal apoC-III mRNA expression and enhances fat clearance to a lesser extent than observed that in apoC-III -/- mice. Chow fed C57BL/6 mice or apoC-III -/- mice were administered 12.5 mg/kg/wk of either a control or murine specific apoC-III ASO for six weeks. A, ApoC-III mRNA expression in the proximal third of the small intestine was quantitated by qPCR. B, Postprandial TG clearance was quantitated by administering a fat bolus to fasted mice, measuring plasma TG concentrations every hour for four hours, and then calculating the plasma TG AUC. *Denotes significant difference (p<0.05) from control ASO.
Online Figure V.

The human apoC-III ASO, ISIS 304801, reduced hepatic apo-CIII mRNA/protein levels and decreased postprandial TG excursion without increasing liver TG deposition in hypertriglyceridemic rhesus monkeys after 10-12 week treatment. Monkeys (n=5 per group) that were made hypertriglyceridemic with fructose supplementation, were treated weekly with 10, 20 or 40 mg/kg/wk of ISIS 304801 or PBS for 12 weeks and at study termination A, Hepatic apoC-III mRNA expression was measured by qPCR and B, plasma apoC-III (mg/dL) was quantified on a clinical analyzer using a commercially available turbidometric assay. C, A postprandial TG excursion test was performed in a separate cohort of rhesus monkeys (n=5 per group) that were treated with a total weekly dose of 10 mg/kg ISIS 304801 or PBS for 10 weeks. The postprandial plasma TG AUC was derived after 0, 1,2,3, and 4 hour plasma collection. D, Liver TG (µg/mg) levels were compared in the 10, 20 and 40 mg/kg/wk cohorts to PBS treated animals after 12 weeks of treatment. For the liver mRNA and TG results data are plotted as mean ± SD. For the plasma apoC-III protein concentrations and TG AUC histograms the mean ± SEM are plotted. *Denotes significant difference from the PBS cohort using one way ANOVA post hoc Tukey’s multicomparison test (p<0.05).
Online Figure VI

FPLC plasma lipoprotein cholesterol and TG analysis.
Representative profiles of A, chow and Western diet fed, C57BL/6 and B, Ldlr -/- mice. Mice treated with 50 mg/kg/wk of either control ASO or apoC-III ASO for 4 weeks were fasted for four hours and plasma was collected. Plasma was then separated by FPLC as described in materials and methods and fractions were analyzed for cholesterol and TG concentrations.