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Letter to Editor

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Is apoCIII-Lowering A Double-Edged Sword?

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Apolipoprotein CIII (apoCIII) is a small protein with 79 amino acid residues which was discovered 50 years ago[1]. ApoCIII contains two amphipathic helices and is mainly synthesized in the liver. ApoCIII is a constituent component of almost all the plasma lipoproteins including chylomicrons, VLDLs, and HDLs and its major physiological function is to mediate plasma triglyceride (TG) homeostasis[2, 3]. Clinically, high levels of plasma apoCIII are positively correlated with plasma TG and TG-rich lipoprotein (TRL) levels and increase cardiovascular risk[4-6]. Our previous study demonstrated that reduced apoCIII expression in knock-out (KO) rabbits protects against high-cholesterol-diet-induced hyperlipidemia and atherosclerosis[7]. Therefore, therapeutically reducing plasma apoCIII is a new strategy for treatment of hypertriglyceridemia and possible prevention of atherosclerosis[8-12]. Until now, two antisense oligonucleotide drugs (volanesorsen and AKCEA-APOCIII-LRx) developed by Ionis Pharmaceuticals, Inc. (Carlsbad, CA) have been approved to treat patients with extremely high hypertriglyceridemia[13]. Volanesorsen is now prescribed in several European countries but has not been approved in the US and Japan, possibly due to concerns about its side-effects as it has been reported that volanesorsen causes platelet reduction, injection site reactions and flu-like symptoms[11]. AKCEA-APOCIII-LRx, another apoCIII antisense oligonucleotide drug that is coupled to a GalNAc moiety, improves specific uptake in the liver and increases potency of inhibiting apoCIII synthesis compared with volanesorsen[12]. Clinical trials (a phase 1/2a) showed that AKCEA-APOCIII-LRx markedly reduced plasma TG levels and apoB levels; however, it has not been determined whether cardiovascular disease endpoint trials will be implemented[13]. Although apoCIII-lowering may become a new therapeutic option for treatment of patients with hypertriglyceridemia, it is still unknown whether this strategy will be beneficial to all hyperlipidemic patients. One concern is whether increased uptake of TRLs in the liver may be detrimental to patients who have impaired liver functions such as those with obese and type 2 diabetes mellitus (T2DM). Can enhanced hepatic uptake of TRLs lead to over-accumulation of lipids in the hepatocytes in the setting of obesity or T2DM? To examine this hypothesis, we took advantage of apoCIII KO rabbits generated in our laboratory[7] and performed the following study (see supplemental materials and methods). ApoCIII KO rabbits do not show any hepatic abnormalities when they are fed a normal regular diet. To increase plasma TRL levels, we fed apoCIII KO and wild-type (WT) rabbit a diet containing 30% fructose (lipogenic sugar) and 10% coconut oil (made up of about 90% saturated fats and 9% unsaturated fats) for 22 weeks[14]. During the experiment with the high fructose and fat diet (HFFD), we measured plasma lipids, glucose, insulin, and performed an intravenous glucose tolerance test. We sacrificed the rabbits and examined the liver and adipose tissue.

Body weight of apoCIII KO rabbits was lighter than WT rabbits at the beginning of the experiment until 6 weeks; however, they gained body weight gradually thereafter even though both groups consumed similar amount of the HFFD diet (g/kg BW/day) **(Fig. 1A)**. After HFFD feeding, as expected, apoCIII KO rabbits showed constantly and significantly lower levels of plasma TG, total cholesterol and FFA compared with WT rabbits **(Fig. 1B)**, suggesting that apoCIII deficiency enhances the catabolism of TRLs in the liver[14]. HFFD feeding

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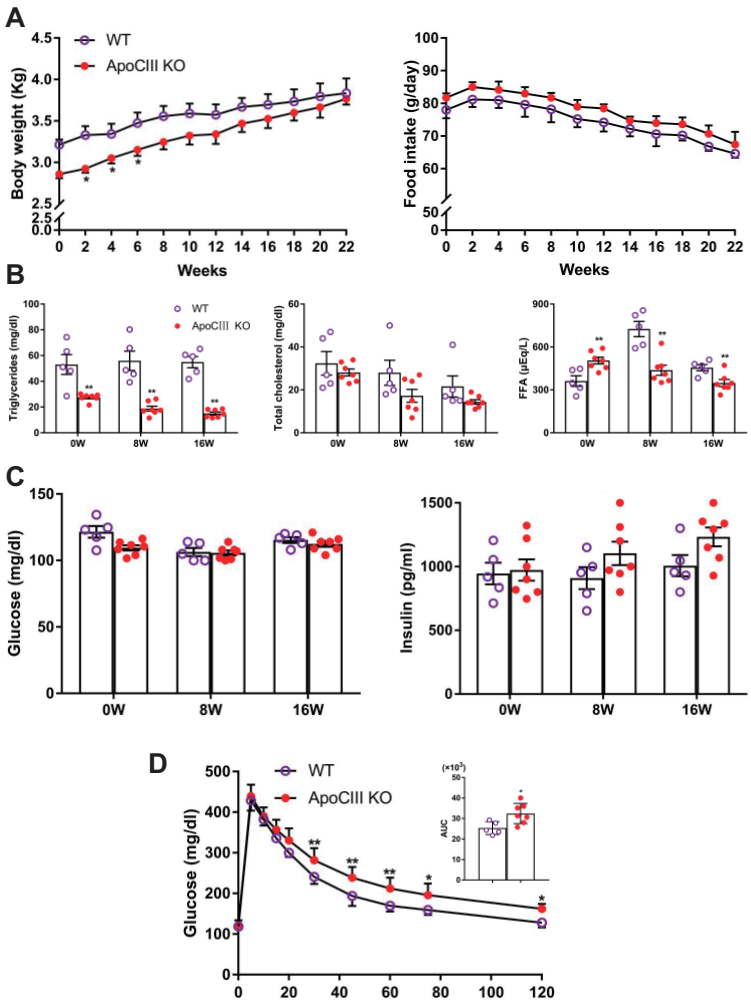

Fig. 1. The changes in body weight and food intake (A), plasma triglycerides, total cholesterol and free fatty acids (FFA) (B), plasma glucose and insulin (C), and glucose metabolism by IVGTT as well as area under the curve (AUC) inserted (D) of 18-week-old male apoCIII KO and WT rabbits after HFFD feeding.

Data are expressed as mean ± SE. $n = 5$ and 7 for WT and KO group, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WT group.

did not change fasting plasma glucose levels in both the apoCIII KO and WT rabbits; however, insulin levels of apoCIII KO rabbits were higher than WT rabbits **(Fig. 1C)**. The hyperinsulinemia exhibited in apoCIII KO rabbits suggests that apoCIII deficiency may be associated with impaired insulin sensitivity which was supported by the finding that apoCIII KO rabbits exhibited delayed glucose clearance compared with WT rabbits by the intravenous glucose tolerance test **(Fig. 1D)**.

After sacrificing, we found that the livers of apoCIII KO rabbits were paler in color and 1.6-fold heavier than those of WT rabbits **(Fig. 2A)**. Histopathological examinations revealed that the liver of apoCIII KO rabbits was characterized by prominent lipid accumulation in the hepatocytes in the pericentral zone. The hepatocytes often contained one or more large lipid droplets, which displaced the nucleus to an eccentric position **(Fig. 2B)**. Quantitation of lipid droplets by oil red O staining showed that hepatic lipids were increased by 11-fold in apoCIII KO rabbits compared with WT rabbits **(Fig. 2C)**. In some areas, lipids were presumably released from ruptured hepatocytes where there were many macrophages or multinucleated macrophages that were present phagocytosing lipids (**Fig. 3**, upper panel). Immunohistochemical staining showed that macrophages in the liver were increased by 8-fold in apoCIII KO rabbits (**Fig. 3**, bottom panel).

In addition to the liver, the volume of visceral

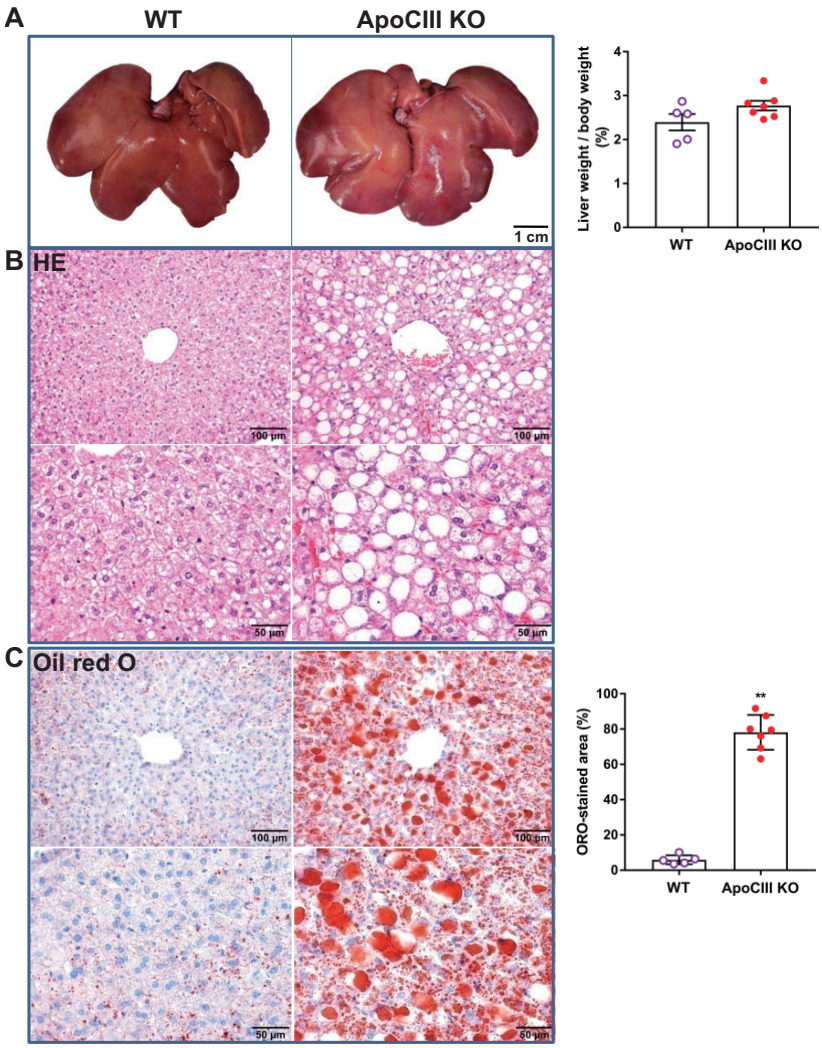

Fig. 2. Pathological examinations of the liver

Representative gross pictures are shown and liver weight is shown on the right and expressed as liver weight/body weight (A). Liver histological examinations were performed using paraffin-embedded specimens stained with HE (B). KO rabbit livers showed prominent lipid droplets in the hepatocytes.
Hepatocyte lipids were quantified using frozen sections stained with oil red O staining. Lipid deposition was expressed as oil red O stained areas under the light microscopy (C). Data are expressed as mean ± SE. $n = 5$ and 7 for WT and KO group, respectively. $^{**}p < 0.01$ vs WT group.

adipose (but not subcutaneous adipose tissue) was increased by 15% in apoCIII KO rabbits compared with WT rabbits although not statistically significant (Fig. 4A). Moreover, histological examinations revealed that adipose tissue of apoCIII KO rabbits was characterized by an increased number of large-sized adipocytes, which leads to the cell size distribution being shifted towards a large size population in both visceral and subcutaneous regions (Fig. 4C). The average diameter of adipocytes in these areas was bigger in apoCIII KO rabbits than in WT rabbits (Fig. 4C). Furthermore, we evaluated the infiltration of macrophages in the adipose tissue and found that the number of infiltrating macrophages was increased in both visceral ($p < 0.05$) and subcutaneous regions (n.s.) of apoCIII KO rabbits (Fig. 5). These macrophages often surrounded an adipocyte and formed so-called "crown-like" structures (Fig. 5).

Our study suggests that apoCIII deficiency indeed enhances TRL catabolism in the liver but simultaneously increases lipid accumulation in the hepatocytes, which leads to steatosis and increased visceral adipose accumulation. Furthermore, the findings of normoglycemia and hyperinsulinemia indicate that apoCIII KO rabbits suffered from insulin resistance when fed a HFFD. There are several

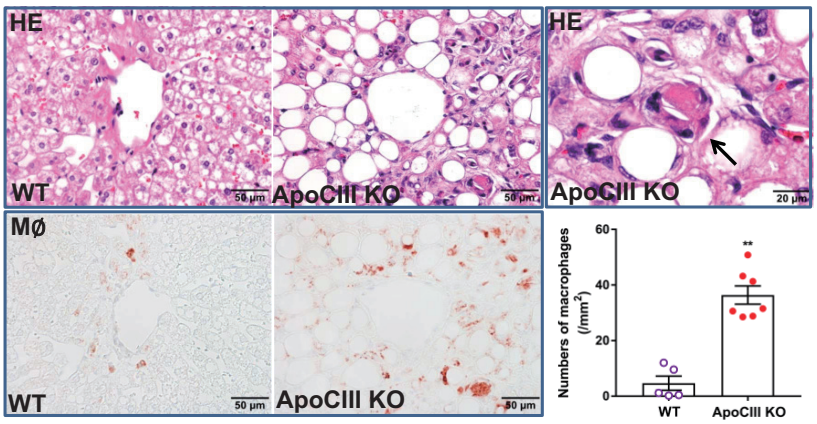

Fig. 3. Increased macrophages in the liver of apoCIII KO rabbits

Representative micrographs stained with HE or immunohistochemically stained with RAM11 antibody against rabbit macrophages (Mϕ) and positive staining areas were quantitated. Data are expressed as mean ± SE. $n = 5$, 7 for WT and KO group, respectively. $^{**}p < 0.01$ vs WT group.

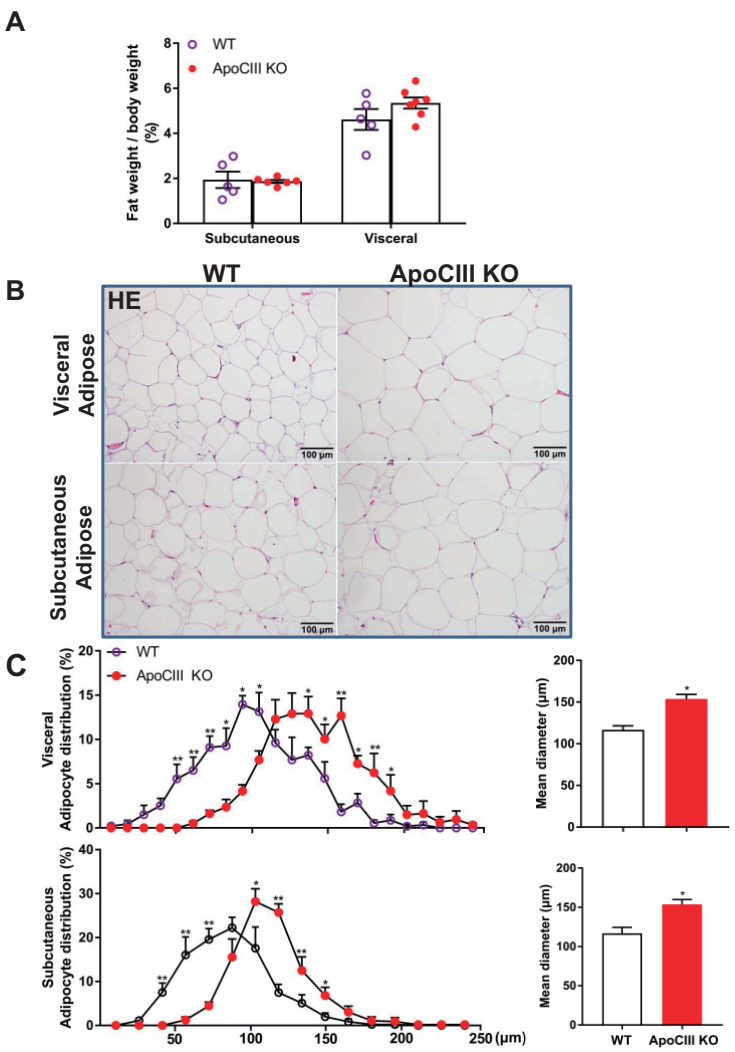

Fig. 4. Adipose tissue changes in apoCIII KO and WT rabbits after HFFD feeding

A. The adipose tissue was measured and expressed as adipose tissue weight to body weight. B. Representative micrographs of adipose stained with HE. C. Adipocyte cell size distribution and mean diameter was measured. Data were expressed as mean ± SE. $n = 5$ and 7 for WT and KO group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WT group.

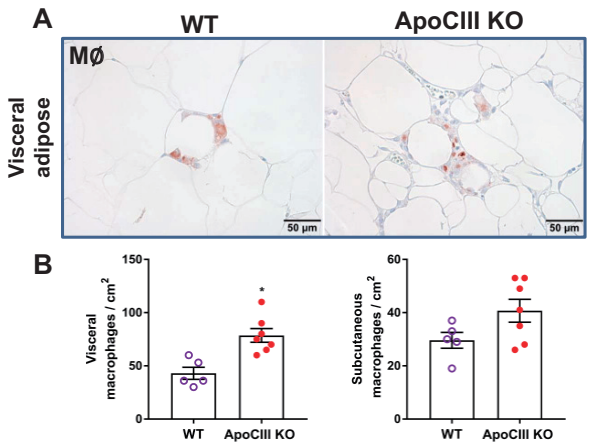

Fig. 5. Demonstration of adipose macrophage infiltration by immunohistochemical staining using RAM11 Ab (A). Positive stained cells were calculated (B).

Data were expressed as mean ± SE. $n = 5$ and 7 for WT and KO group. *$p < 0.05$ vs WT group.

possible mechanisms for insulin resistance. For example, both fatty liver and visceral adipose tissue accumulation simultaneously induce insulin resistance[15] and an increased number of large-sized adipocytes reduces the sensitivity of insulin compared with small-sized adipocytes[16]. Although it is not known whether apoCIII is involved in the secretion of insulin or insulin signaling[17-19], both high and low levels of apoCIII were paradoxically associated with insulin resistance. Nevertheless, the current study suggests that caution should be taken when treating patients with obese, nonalcoholic fatty liver disease, and T2DM using apoCIII-lowering therapies. In the future, further studies are needed to investigate whether apoCIII inhibition has any deleterious effects on the fatty liver of obese and T2DM patients.

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Disclosure

None.

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Supplementary Materials and Methods

1. Animals and Diet

In the current study, we used 18-week-old male apoCIII knockout (KO) and wild-type (WT) NZW rabbits[1]. All the rabbits were fed a normal regular diet supplemented with high-fructose (30%) and high-fat (10% coconuts oil) (designated as HFFD) for 22 weeks[2]. Rabbits were housed in individual cages in the room with controlled temperature on a 12h light/dark cycle. The body weight and food consumption were monitored during the experiment. All animal experiments were performed according to the approval of the Animal Care Committee of the University of Yamanashi and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2. Analysis of Plasma Lipids and Glucose Metabolism

Blood samples were collected after fasting for 16 hours, and plasma total cholesterol (TC), triglycerides (TG), and free fatty acids (FFAs) were determined using Wako commercial assay kits (Wake Pure Chemical Industries, Ltd, Osaka, Japan)[3]. In addition, we measured the levels of plasma glucose and insulin and performed the intravenous glucose tolerance test (IVGTT) at 17 weeks after HFFD feeding[4]. In brief, the rabbits were injected with glucose solution (0.6 g/kg body weight) intravenously after 16h of fasting, and then the blood samples were collected at 5, 10, 15, 20, 30, 45, 60, 75, and 120min. The concentrations of glucose and insulin were measured using Wako glucose assay kits and rabbit insulin ELISA kits (Shibayagi Co., Ltd., Gunma, Japan).

3. Pathological Examinations

All the animals were sacrificed by an overdose injection of sodium pentobarbital (100mg/kg). Subcutaneous adipose (scapular and inguinal adipose) and visceral adipose (abdominal, mesenteric, and epididymal regions) along with the liver were collected and weighted wet[3]. For histological examinations, we collected pieces of inguinal and mesenteric adipose and liver and fixed them in 10% buffered formalin. Then, these specimens were embedded in paraffin and sections (3μm) were cut and stained with hematoxylin-eosin (H&E). To evaluate the lipid accumulation in the liver, we performed oil red O staining. A piece of fresh liver in Tissue-Tek® O.C.T. compound was quickly embedded in liquid nitrogen and frozen sections (7μm) were made using a Leica cryostat and stained with oil red O.

Adipocyte size was evaluated under a light microscope using H&E-stained sections as previously[5]. In brief, the cellular diameters of 300 adipocytes from each section were randomly measured by two observers blindly with an image analysis system WinRooF V6.4.0 (Mitani Co, Tokyo, Japan). The average diameter of adipocytes in each group was calculated and the cell size distribution was analyzed and expressed as a percentage.

Immunohistochemical staining was used to evaluate the macrophage infiltration in the liver and adipose. Briefly, paraffin-sections were deparaffinized with xylene and ethanol and immersed in 3% hydrogen peroxide (H_2O_2) methanol for 10 minutes to block the endogenous peroxidase activity. Specimens were further treated in citric buffer solution and followed by antigen retrieval solution (pH9.0) (Nichirei Bioscience, Tokyo, Japan) for 10 minutes. After that, these specimens were incubated with the primary monoclonal antibody (mAb) against rabbit macrophage (RAM11, Dako, working dilution, 400x) for 2 hours at room temperature and followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Nichirei Bioscience) for 1 hour at room temperature. Amino-9-ethylcarbazole (AEC) (Nichirei Bioscience) was used as a substrate for visualizing the antigen signals and nuclei were stained with haematoxylin.

To evaluate Ab specificity, the slides were incubated with mouse non-specific IgG or PBS to replace the first Ab. The number of macrophages stained by RAM11 was quantified by counting each section under 10 high power fields and expressed as the total number per μm^2.

4. Statistical Analysis

All data were expressed as the mean ± SEM. Data were analyzed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). The Shapiro-Wilk test was used to verify the normal distribution of all data. The Student's *t*-test was used. *P*-values less than 0.05 were considered significant.

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