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## **The role of ApoCI, LPL and CETP in plasma lipoprotein metabolism - studies in mice**

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## **ApoE\*2-Associated Hyperlipidemia is Ameliorated by Increased Levels of ApoAV, but Unaffected by ApoCIII-Deficiency**

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*Submitted*

**Objective** - ApoE\*2-associated hyperlipidemia is characterized by a disturbed clearance of apoE\*2-enriched VLDL-remnants. Since excess apoE\*2 inhibits lipoprotein lipase (LPL)-mediated triglyceride (TG)-hydrolysis *in vitro*, we investigated whether direct or indirect stimulation of LPL activity *in vivo* reduces the apoE\*2-associated hyperlipidemia.

**Methods and Results** - Hereto, we studied the role of LPL and two potent modifiers, the LPL-inhibitor apoCIII and the LPL-activator apoAV in *APOE\*2*-knockin (*APOE\*2*) mice. Injection of heparin in *APOE\*2* mice reduced plasma TG by 55% and plasma total cholesterol (TC) by 28%. Similarly, adenovirus-mediated overexpression of LPL reduced plasma TG by 85% and TC by 40%, indicating that apoE\*2-enriched particles can serve as substrate for LPL. Indirect activation of LPL activity via deletion of apoCIII in *APOE\*2* mice did neither affect plasma TG nor TC levels, whereas overexpression of *Apoa5* did reduce plasma TG by 81% and plasma TC by 41%.

**Conclusion** - In conclusion, the combined hyperlipidemia in *APOE\*2* mice can be ameliorated by direct activation of LPL activity. Indirect activation of LPL via overexpression of apoAV does, whereas deletion of apoCIII does not affect the lipid phenotype of *APOE\*2* mice. These data indicate that changes in apoAV levels have a dominant effect over changes in apoCIII levels in the improvement of *APOE\*2*-associated hyperlipidemia.

ApoE\*2-associated hyperlipidemia is characterized by increased plasma levels of chylomicron and VLDL remnants and is associated with xanthomatosis and premature atherosclerosis.<sup>1</sup> ApoE\*2 has a single amino acid substitution (Arg158 to Cys) as compared with the common apoE3 variant, resulting in a low binding affinity for the LDLR.<sup>2,3</sup> *In vivo*, this is associated with impaired hepatic clearance of VLDL and chylomicron remnant particles,<sup>4</sup> resulting in increased plasma TG and TC levels. Simultaneously, apoE\*2 accumulates in plasma leading to an increase in apoE-mediated inhibition of LPL-mediated TG hydrolysis.<sup>5</sup> It has been postulated that both impaired remnant clearance and impaired remnant generation via lipolysis contribute to the hyperlipidemia associated with apoE\*2.<sup>5</sup>

We and others have found that VLDL obtained from hyperlipidemic patients homozygous for APOE\*2 is a relatively poor substrate for LPL-mediated lipolysis.<sup>6</sup> Two potent modifiers of LPL activity have been described, apoAV and apoCIII, that are encoded in same gene cluster on chromosome 11.<sup>7</sup> *In vitro* and *in vivo* mouse studies indicate that apoAV stimulates LPL-mediated TG hydrolysis and that apoCIII inhibits this process.<sup>8-12</sup> Overexpression of apoAV in mice reduces plasma TG levels via stimulation of LPL activity<sup>13</sup> and overexpression of apoCIII results in increased plasma TG levels via inhibition of LPL.<sup>14</sup> Studies in *ApoC3*-knockout mice show accelerated LPL-mediated TG hydrolysis.<sup>15,16</sup> Deficiency in apoAV in both mice and humans is associated with hypertriglyceridemia.<sup>17-19</sup>

In the present study, we have investigated the role of LPL-mediated TG-hydrolysis in apoE\*2 associated hyperlipidemia *in vivo*. Direct stimulation of LPL activity in APOE\*2 knockin (APOE\*2) mice via heparin injection and via adenovirus mediated gene transfer of LPL both reduced the TG and TC levels. Indirect stimulation of the LPL activity via deletion of endogenous *ApoC3* did not affect the lipid levels, whereas indirect stimulation via adenovirus mediated overexpression of apoAV did result in decreased plasma TG and TC levels. Thus, stimulation of LPL activity via apoAV overexpression or deficiency of apoCIII occur via different mechanisms. Moreover, these data indicate that apoAV represents a potential target for the improvement of APOE\*2 associated hyperlipidemia.

## Methods

### *Adenoviral Constructs*

The adenoviral vector expressing active LPL (AdLPL)<sup>20</sup> was kindly provided by Dr. Santamarina-Fojo. The generation of the adenoviral vectors expressing apoAV (AdApoa5), the control empty vector (AdEmpty) and  $\beta$ -galactosidase (AdLacZ) have been described.<sup>8,13</sup> Expansion, purification and titration of the adenoviral vectors were performed as described previously.<sup>21</sup> Before *in vivo* administration, the adenoviral vectors were diluted to a dose of  $5 \times 10^8$  pfu in 200  $\mu$ l sterile PBS.

### *Mouse Models*

APOE\*2 knockin mice, carrying the human APOE\*2 gene in place of the mouse *ApoE* gene have been described previously.<sup>22</sup> These mice were backcrossed 8 times with

C57Bl/6 mice to achieve a more homogenous genetic background and subsequently intercrossed to obtain homozygous *APOE\*2* mice. *Apoc3*<sup>-/-</sup> mice were obtained from The Jackson Laboratories (Bar Harbor, ME, USA) and intercrossed with *APOE\*2* mice to obtain *APOE\*2*, *APOE\*2.Apoc3*<sup>+/-</sup> and *APOE\*2.Apoc3*<sup>-/-</sup> mice. The mice were fed a regular mouse diet (SRM-A: Hope Farms, Woerden, The Netherlands) and given free access to food and water. At least five days before adenovirus injection, mice were transferred to filter-top cages in designated rooms. All animal experimentation protocols were approved by the Committee on Animal Experimentation of the Leiden University Medical Center.

#### *Adenovirus-Mediated Gene Transfer in Mice*

Male *APOE\*2* mice at the age of 13-18 weeks were selected for injection with Ad*LPL*. A dose of  $5 \times 10^8$  pfu adenovirus was injected into the tail vein. Prior to and 5 days after administration of Ad*LPL*, mice were fasted for 4 h and a blood sample for lipid determination was collected by tail bleeding, using diethyl-p-nitro phenyl phosphate (paraoxon, Sigma) coated heparinised capillary tubes (Hawksley, Sussex, England).

Female *APOE\*2* mice between the age of 13 and 18 weeks were injected with a dose of  $5 \times 10^8$  pfu of Ad*Apoa5* or  $5 \times 10^8$  of empty vector (AdEmpty). Three hours prior to this virus injection, the mice were injected with  $5 \times 10^8$  pfu Ad*LacZ* to saturate the uptake of viral particles by hepatic Kupffer cells.<sup>23</sup> Prior to injection and 4 days after virus injection, mice were fasted for 4 h and a blood sample for lipid determinations was collected in paraoxon-coated capillaries by tail bleeding.

#### *Lipid Determinations*

Plasma was isolated from blood samples obtained from the mice by centrifugation. TG and TC levels were measured enzymatically (Sigma). Human apoE levels were measured by sandwich ELISA as described previously.<sup>24</sup> The circulating human apoE level in homozygous *APOE\*2* carrying mice was  $3.1 \pm 0.9$  mg/dl.

Lipoprotein fractions were separated using fast protein liquid chromatography (FPLC). Hereto, a plasma pool obtained from the groups of mice were diluted 5 times using PBS. A volume of 50  $\mu$ l was injected onto a Superose 6 column (3.2 x 30 mm, Äkta System, Pharmacia, Uppsala, Sweden) to separate lipoprotein fractions. Elution fractions of 50  $\mu$ l were collected and assayed enzymatically for TG and TC levels as described above.

#### *Heparin Treatment*

Heparin was administered to *APOE\*2* mice after a period of 4 hours fasting and via *i.v.* injection of a dose of 0.5 U/g body weight. Blood samples of approx. 30  $\mu$ l were drawn via the tail vein at  $t = 0, 10, 30, 60$  and 120 minutes after heparin injection, using paraoxon coated capillaries. Plasma TG levels were measured enzymatically, as described above.

### Fat-Load

The fat-load response was determined in male *APOE\*2*, *APOE\*2.ApoC3<sup>+/-</sup>* and *APOE\*2.ApoC3<sup>-/-</sup>* mice aged 13 to 20 weeks. The mice were fasted over night and given an intra-gastric olive oil load (Carbonell, Cordoba, Spain) of 400  $\mu$ l. Prior to the olive oil load and 3 and 6 h after the load, a blood sample was drawn via the tail vein for TG determination. The circulating levels were corrected for the TG level prior to the fat-load. The Area Under the Curve (AUC) was determined over the period of 6 h.

### Statistical Analysis

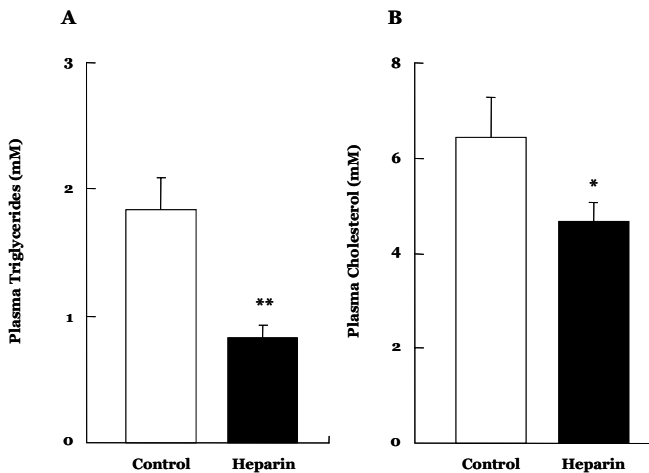
Data were analyzed using the non-parametric Mann-Whitney U test. *P*-values less than 0.05 were regarded as statistically significant.

## Results

### Effect of Increased LPL Activity on Lipid Levels in APOE\*2 Mice

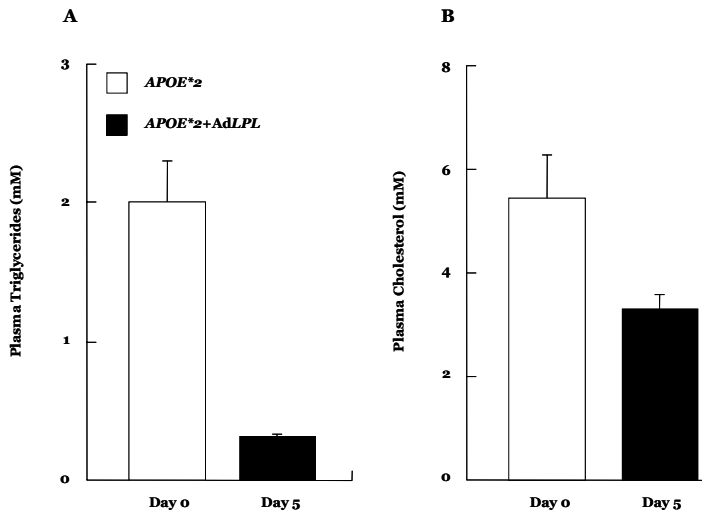
*I.v.* injection of heparin results in activation of LPL and its release from the endothelial surfaces. Stimulation of LPL activity in *APOE\*2* mice via injection of heparin reduced the hyperlipidemia (Fig. 1). The maximum reduction was observed at 60 minutes after injection of 0.5 U heparin/g body weight. The plasma TG levels decreased 55% ( $P<0.005$ ,  $n=4$ ). The TC levels in *APOE\*2* mice decreased 28% ( $P<0.05$ ,  $n=4$ ).

*APOE\*2* mice were injected with adenovirus expressing LPL to determine the effect on hyperlipidemia (Fig. 2). At day 5 after injection of  $5 \times 10^8$  pfu AdLPL, *APOE\*2*



**Figure 1. Plasma lipid levels of APOE\*2 mice after heparin treatment.** Fasted *APOE\*2* mice were injected with heparin. Before (open bars) and 1 hour after injection (black bars), plasma samples were obtained and assayed for triglyceride (A) and cholesterol (B). The values are represented as means  $\pm$  SD for  $n=4$  mice per group. \* $P<0.05$ , \*\* $P<0.005$ .

mice exhibited a 85% decrease in plasma TG levels (n=3). The TC levels decreased 40% (n=3). The lipoprotein distribution as determined by FPLC showed a decrease in VLDL-TG and VLDL-TC to wild type levels after injection of AdLPL, indicating an accelerated conversion of *APOE\*2*-containing VLDL particles by overexpression of LPL (data not shown).

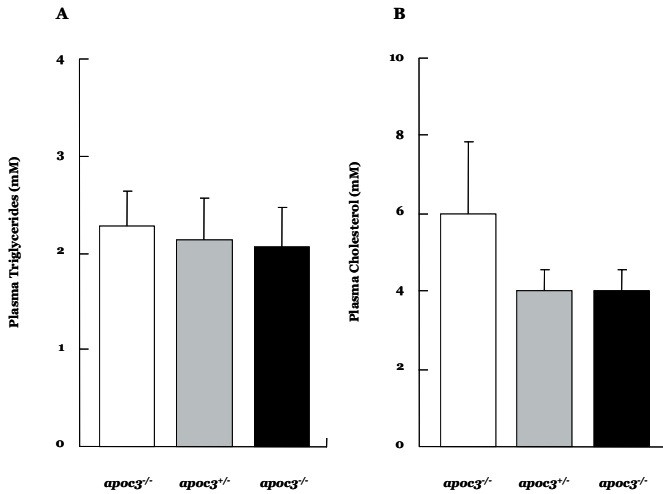


**Figure 2. Plasma lipid levels of *APOE\*2* mice injected with AdLPL.** *APOE\*2* mice were injected with  $5 \times 10^8$  pfu AdLPL. Before (open bars) and at day 5 after adenovirus injection (black bars), fasted plasma samples were assayed for triglyceride (A) and cholesterol (B). Values are represented as means  $\pm$  SD for n=3 mice per group

### *Effect of ApoCIII-Deficiency on Lipid Levels in *APOE\*2* Mice*

The main endogenous inhibitor of LPL, apoCIII, was deleted from the genetic background of *APOE\*2* mice by crossbreeding with *Apoc3* knockout mice. The effect of *Apoc3*-deficiency on *APOE\*2*-associated hyperlipidemia was investigated in *APOE\*2* mice heterozygous or homozygous deficient for the endogenous *Apoc3* gene (Fig. 3). Surprisingly, the plasma TG levels were not different between *APOE\*2*, *APOE\*2.Apoc3<sup>+/-</sup>* and *APOE\*2.Apoc3<sup>-/-</sup>* mice. Also, the TC levels were not affected by *Apoc3*-deficiency in the presence of *APOE\*2*. No differences in plasma lipid levels were found between male and female mice (data not shown). The distribution of TG and TC over the lipoprotein fractions was measured after separation via FPLC. No differences were observed between *APOE\*2*, *APOE\*2.Apoc3<sup>+/-</sup>* and *APOE\*2.Apoc3<sup>-/-</sup>* mice (data not shown).

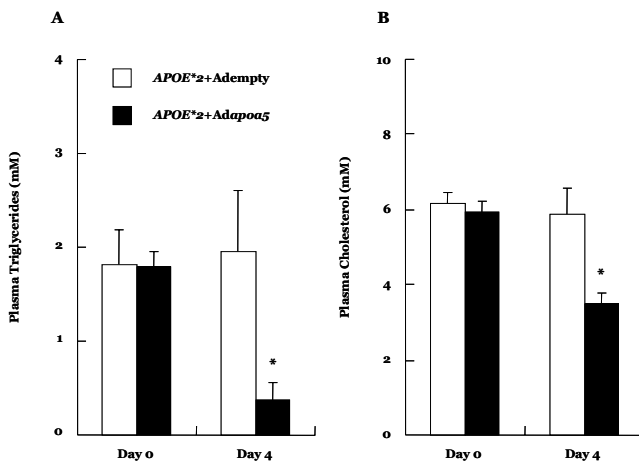
To further analyse the effect of apoCIII-deficiency in *APOE\*2* mice on TG metabolism, mice were given an intragastric olive oil load. The increase in plasma TG levels were measured over a period of 6 h and the AUC was determined. The response in *APOE\*2* carrying mice was not different (*APOE\*2* AUC 5.8; *APOE\*2.Apoc3<sup>+/-</sup>* AUC 5.5 and *APOE\*2.Apoc3<sup>-/-</sup>* mice AUC 4.3 mM/6 h, n.s. for n=5 mice per group, data not shown).



**Figure 3. Plasma lipid levels of APOE\*2 mice deficient for Apoc3.** Fasted plasma samples were obtained from APOE\*2 mice (open bars), APOE\*2.ApoC3<sup>+/-</sup> mice (hatched bars) and APOE\*2.ApoC3<sup>-/-</sup> mice (black bars). The samples were assayed for triglycerides (A) and cholesterol(B). Values are represented as mean ± SD for n=5 mice per group .

*Effect of Adenovirus-Mediated Expression of ApoA5 on Lipid Levels in APOE\*2 Mice.*

The activator of LPL, apoAV, was expressed in APOE\*2 mice via a recombinant adenoviral vector. Injection of a moderate dose of AdApoa5 (5x10<sup>8</sup> pfu) reduced plasma TG by 81% (*P*<0.05) and TC by 41% (*P*<0.05) as compared to AdEmpty (Fig. 4). Analysis of lipoprotein fractions separated by FPLC revealed that the apoAV-mediated reduction of plasma TG was associated with a 4-fold reduction in VLDL-TG, whereas the TG level in the IDL/LDL fraction was affected to a minor degree. The reduction in plasma TC level was associated with a 2-fold reduced VLDL-TC level (data not shown).



**Figure 4. Plasma lipid levels of APOE\*2 mice injected with AdApoA5.** APOE\*2 mice (n=5 per group) were injected consecutively with AdLacZ (5x10<sup>8</sup> pfu) and AdApoa5 or AdEmpty (5x10<sup>8</sup> pfu). Before injection (open bars) and at 4 days after injection (black bars), fasted plasma was collected from the individual mice and assayed for triglyceride (A), cholesterol (B). Values are represented as means ± SD. \**P*<0.05.



## Discussion

In the current study, we have addressed the hypothesis that alleviating the apoE\*2-mediated inhibition of lipolysis can reduce the apoE\*2-associated hyperlipidemia. Using the *APOE\*2* mouse model, we first stimulated LPL activity directly via heparin injection, which releases and activates endogenous LPL. This resulted in a reduction of the TG and TC levels in *APOE\*2* mice (Fig. 1). Likewise, injection of adenovirus expressing LPL in *APOE\*2* mice reduced the plasma TG and TC levels (Fig. 2). The reduction in TG and TC was mainly confined to the VLDL-sized fractions (data not shown). Subsequently, LPL was stimulated indirectly via its oppositely acting modulators apoCIII and apoAV. *Apoa5* overexpression did reduce the *APOE\*2*-associated hyperlipidemia in *APOE\*2* knock-in mice (Fig. 4). In contrast, the *APOE\*2*-associated hyperlipidemia was not affected by *Apoc3*-deficiency (Fig. 3). Our data indicate that a direct increase of LPL activity by increasing circulating LPL levels reduces *APOE\*2* associated hyperlipidemia. The indirect stimulation of LPL activity via apoAV overexpression but not apoCIII-deficiency ameliorates the *APOE\*2*-associated hyperlipidemia. We conclude that apoAV is apparently dominant over apoCIII in the improvement of *APOE\*2*-associated hyperlipidemia. Moreover, apoAV and apoCIII modulate LPL activity via distinct mechanisms.

Addition of apoE to lipoproteins results in a decrease in the LPL-mediated TG hydrolysis.<sup>25-27</sup> This can at least partially explain the hypertriglyceridemia that is found in *APOE\*2*-associated familial dysbetalipoproteinemia (FD), which is characterized by plasma accumulation of apoE-enriched lipoproteins. It has been proposed that inhibition of LPL activity is caused by displacement of the LPL-coactivator apoCII from the apoE\*2-rich lipoprotein particles.<sup>5</sup> However, this is difficult to reconcile with the observation that indirect stimulation of LPL activity via apoAV overexpression ameliorates the *APOE\*2*-associated hyperlipidemia. Especially, since it has been demonstrated that the LPL-activating effect of apoAV is dependent on the presence of apoCII.<sup>8</sup> Thus other mechanisms might underlie the inhibitory effect of apoE\*2 on LPL activity.

Under normal conditions, LPL-mediated TG hydrolysis takes place mainly at the endothelial cell surface and may thus be affected by the interaction between the TG-containing particle and the cell surface where LPL is localized. This interaction involves the association of TG-rich particles and endothelial surface bound heparan sulfate proteoglycans (HSPG) via apoE.<sup>28</sup> It has been shown that apoE\*2 is partly defective in the association with HSPG<sup>29</sup> and this could also explain part of the apoE\*2-associated hypertriglyceridemia. In agreement with this hypothesis, it has been found *in vitro* that VLDL obtained from *APOE\*2* homozygous FD patients is effectively lipolysed by LPL in solution, but poorly lipolysed by HSPG-bound LPL.<sup>6</sup> Thus, apoE\*2-containing VLDL may be defective in the physical association with the endothelial surfaces where LPL-mediated TG hydrolysis takes place *in vivo*. This would explain why additional LPL via adenovirus mediated gene transfer and endothelial release and activation of endogenous LPL by heparin do rescue the apoE\*2-associated hyperlipidemia. Intriguingly, this explanation is also in line with the observation that additional apoAV rescues the apoE\*2-associated hyperlipidemia. It has recently been found that the LPL-activating

effect of apoAV involves enhanced binding to HSPG.<sup>10,30</sup> Thus additional apoAV on the TG-rich particle apparently overcomes the apoE\*2-mediated inhibition of HSPG binding. It is interesting to note that apoCIII-deficiency cannot overcome this binding defect, despite postulated inhibition of HSPG-binding by apoCIII.<sup>14,31</sup> However, the *in vivo* contribution of HSPG in the lipolysis of TG-rich lipoprotein particles still remains to be determined.

The AdLPL and heparin-induced decrease in plasma TG levels was accompanied by a decrease in TC levels. This is likely due to increased clearance of TC and can be explained by two mechanisms. First, stimulation of LPL-mediated processing of VLDL and chylomicrons will lead to accelerated generation of remnant particles that are more easily cleared by the liver. Second, the AdLPL and heparin induced increase in the pool of LPL may result in enhanced binding of apoE\*2-containing lipoproteins to the liver via an LPL-mediated bridging effect.<sup>32</sup> This would result in enhanced hepatic clearance of whole particles and thus a reduction in both plasma TG and TC. Whether one or both of these mechanisms play a dominant role in mediating the hypocholesterolemic effect of AdLPL and heparin remains to be determined.

Previously, we have shown that *Apoc3*-deficiency is a potent tool to accelerate LPL-mediated TG-hydrolysis and to reduce the severe combined hyperlipidemia induced by adenovirus-mediated overexpression of *APOE4*.<sup>16</sup> This hyperlipidemia is caused by an apoE4-induced increase in VLDL-production and simultaneous apoE4-mediated inhibition of VLDL-TG lipolysis.<sup>33</sup> Despite a 10-fold increase in VLDL-TG production rate in Ad*APOE4* treated mice, *Apoc3*-deficiency did result in a normalization of circulating lipid levels.<sup>16</sup> To our surprise, *Apoc3*-deficiency did not affect the hyperlipidemia or lipoprotein lipid distribution (data not shown) in *APOE\*2* mice. Moreover, stressing the TG metabolism by an intragastric bolus injection of olive oil also did not induce a different post prandial TG response in *APOE\*2* mice on *Apoc3* deficient or wild-type backgrounds. The absence of a hypolipidemic effect of *Apoc3*-deficiency in *APOE\*2* mice indicates that the defect in *APOE\*2*-associated hyperlipidemia is upstream from the positive effect associated with apoCIII deficiency.

Apart from a stimulatory effect on LPL, the decrease in plasma TG of *APOE\*2* mice after expression of apoAV may have resulted from a decrease in the VLDL-TG secretion rate by the liver. We have previously shown a 30% decreased VLDL-TG secretion rate after adenovirus-mediated overexpression of *Apoa5* in wild-type C57Bl/6 mice,<sup>8</sup> whereas others have found no effects of apoAV on VLDL production in neither *APOA5* transgenic mice<sup>34</sup> nor in *Apoa5*<sup>-/-</sup> mice.<sup>17</sup> Intriguingly, in the *APOE\*2* mice, we did not observe differences in the VLDL-TG secretion rate between Ad*Apoa5*- or AdEmpty-treated mice (data not shown). At present, we have no explanation for these apparent discrepancies, but cannot exclude that apoAV has additional yet unrecognized functions.

Polymorphisms in both the *APOA5* and *APOC3* genes have been associated with hypertriglyceridemia.<sup>19,35-39</sup> Since both genes are expressed in the same gene cluster and have opposing effects on TG levels, it has been hypothesized that these genes act synergistically.<sup>7,34</sup> Our current data clearly indicate that apoAV and apoCIII affect different steps in the conversion of TG rich lipoproteins to remnants. Moreover, within the con-

text of *APOE*\*2-associated hyperlipidemia, it seems likely that variation in apoAV level and activity will have a more pronounced effect on the expression of hyperlipidemia as compared to variation in apoCIII level and activity.

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