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### Title

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# **Independent Effects of Apolipoprotein AV and Apolipoprotein CIII on Plasma Triglyceride Concentrations**

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**ABSTRACT**

Both the apolipoprotein A5 and C3 genes have repeatedly been shown to play an important role in determining plasma triglyceride concentrations in humans and mice. In mice, transgenic and knockout experiments indicate that plasma triglyceride levels are negatively and positively correlated with *APOA5* and *APOC3* expression, respectively. In humans, common polymorphisms in both genes have also been associated with plasma triglyceride concentrations. The evolutionary relationship among these two apolipoprotein genes and their close proximity on human chromosome 11q23 have largely precluded the determination of their relative contribution to altered triglycerides. To overcome these confounding factors and address their relationship, we generated independent lines of mice that either over-expressed ("double transgenic") or completely lacked ("double knockout") both apolipoprotein genes. We report that both "double transgenic" and "double knockout" mice display intermediate triglyceride concentrations compared to over-expression or deletion of either gene alone. Furthermore, we find that human ApoAV plasma protein levels in the "double transgenic" mice are approximately 500-fold lower than human ApoCIII levels, supporting ApoAV is a potent triglyceride modulator despite its low concentration. Together, these data indicate that *APOA5* and *APOC3* independently influence plasma triglyceride concentrations but in an opposing manner.

## INTRODUCTION

Apolipoproteins constitute a class of polypeptides found on plasma lipoprotein particles that play an important role in lipid transport and metabolism. Alterations in the level or structure of these molecules have been shown to dramatically impact plasma lipid concentrations, and in many cases atherosclerosis susceptibility in humans as well as in mice. In mammals, evidence exists that some of the apolipoprotein family members are evolutionarily related as the result of gene duplication events. For instance, apolipoprotein AI, AIV, and E all share amino acid identity and similarity, supportive of a common ancestral origin (1). We recently identified an additional member of this apolipoprotein family, named ApoAV, through the use of human-mouse genomic sequence comparisons (2). *APOA5* is located within the well-described *APOA1/A4/C3* gene cluster on human chromosome 11q23 (3). While *APOA5* is most closely related to *APOA4*, manipulations of *APOA5* levels in mice resulted in profound effects on plasma triglyceride concentrations, a phenotype not present in *APOA4* mouse models (2, 4, 5). *APOA5* transgenic mice displayed significantly reduced (~70%) triglyceride concentrations while *apoA5* knockouts had a four-fold increase in this lipid parameter (2). Further genetic studies in humans have consistently reported strong association between common *APOA5* polymorphisms and plasma triglyceride concentrations (2, 6-11).

In addition to *APOA5*, the neighboring *APOC3* gene has also been reported to have a striking effect on human and mouse plasma triglyceride concentrations. However, *APOC3* has a opposite impact on triglycerides with transgenic mice having increased concentrations (~200-2000%) and knockout mice having decreased concentrations (~30%) (12, 13). Furthermore,

genetic association studies have supported that common polymorphisms in *APOC3* also contribute to triglyceride abnormalities in humans (14-19).

The inverse effect of *APOA5* and *APOC3* on plasma triglycerides *in vivo* raises the question whether they mediate their effect through either a shared or independent mechanism. One hypothesis is that alteration in *APOA5* or *APOC3* levels in transgenic or knockout mice simply disrupts the plasma protein level of the other apolipoprotein and accounts for the abnormal triglyceride phenotype. For instance, in *APOA5* transgenic mice, apoCIII levels could be reduced by displacement due to the over-expression of *APOA5* and this reduction in apoCIII is the mechanism behind the lower triglycerides (an apoCIII dependent pathway). In contrast, in *APOC3* transgenic mice, apoAIV levels could be reduced due to the over-expression of *APOC3* and this reduction in apoAIV is in fact the mechanism behind the lower triglycerides (an apoAIV dependent pathway). Finally, apoAIV and apoCIII could function independently to modulate triglyceride concentrations in an opposing fashion.

To address this issue, we pursued a genetic approach and generated two independent lines of mice with simultaneous alterations in both ApoAIV and ApoCIII levels. The first line of mice was engineered to completely lack both genes ("double knockouts"), while the second line was created to over-express both genes ("double transgenics"). We hypothesized that if ApoAIV and ApoCIII affect a common pathway in an inter-dependent fashion then the resulting phenotype in the "double knockouts" should be similar to that in either of the single knockouts alone (extremely high or low triglyceride concentrations). In contrast, if apoAIV and apoCIII function independently, then the loss of both genes should result in intermediate triglyceride

concentrations (somewhere between that found in either of the single knockouts alone). A similar logic was applied to the utility of analyzing "double *APOA5/APOC3* transgenic" mice.

## MATERIALS AND METHODS

### Double Knockout Mouse Generation

Double knockout mice were generated by sequential targeting in embryonic stem (ES) cells. ES cells were first targeted for the *apoA5* locus using a modified version of a previously described vector (2). Briefly, pPN2T-apoAV-KO was digested with *XbaI* to remove the neomycin cassette and the remaining vector backbone was end-filled with Klenow and ligated to a *SmaI* hygromycin cassette derived from pGK-hygro (20). 129/SvJ ES cells (Incyte Genomics, Palo Alto, CA) were electroporated with 20  $\mu$ g of *NotI* linearized targeting construct and subsequently selected in 140  $\mu$ g/ml hygromycin and 0.5  $\mu$ g/ml FIAU for 8 days. Individual clones were isolated, expanded and screened for proper targeting by long range PCR analysis. PCR amplification was performed using Platinum-High Fidelity Taq polymerase as recommended by the manufacturer (Invitrogen, Carlsbad, CA) with a 4 minute extension. The two primer pairs used were: 1) apoA5-extnl-F1-5'-TGAGATTGCAGGCACGGGCTAC and pGK-hygro-extnl-R1-5'-AGAATTCATCGATGATCGGGATCC (56°C annealing) and 2) hygro-extnl-F15-CAAATTAAGGGCCAGCTCATTCCCTC and apoA5-extnl-R2-5'-GGTACCAAAGGACACCTCAAAGGAC (60°C annealing). Nine positive colonies were identified, pooled and expanded for subsequent *apoc3* targeting with a previously described vector using a neomycin selection scheme (12). 20  $\mu$ g of *NotI* linearized targeting construct were electroporated and subsequently selected in 175  $\mu$ g/ml G418 and 0.5  $\mu$ g/ml FIAU for 8 days. Properly targeted clones were identified by long range polymerase chain reaction (PCR) amplification as described above with two primer pairs: 1) ApoC3-extnl-F2-5'-

GGCGTGGCCCAACTCTGCTCT-3' and pMC1neo-extnl-R10-5'-TGCTCGACATTGGGTGGAAACAT-3' (60°C annealing) and 2) pMC1neo-extnl-F20-5'-GCTGACCGCTTCCTCGTGCTTTA-3' and ApoC3-extnl-R20-5'-GAGCAGGAGGCAGGAACATCATTC-3' (60°C annealing). Double targeted clones were expanded and injected into C57BL/6 blastocysts and the resulting chimeric males were bred to C57BL/6 females (The Jackson Laboratory, Bar Harbor, MN). Agouti offspring were tested for germline transmission of the targeted alleles by PCR using primers specific to the neomycin and hygromycin genes (neoR1-5'-CAGGCATCGCCATGGGTTCAC-3' and neoF1-5'-GAAGCCGGTCTTGTCGATCAGG-3'; hydroF1-5'-AGCCTGAACTCACCGCGACGT-3' and hydroR1-5'-CAGGACATATCCACGCCCTCCTA-3'). Heterozygous mice were inter-crossed to obtain homozygous deletion animals for the *apoa5* and *apoc3* loci. Offspring were genotyped with PCR primers designed to the neomycin and hygromycin genes (described above) and with primers contained within the *apoa5* and *apoc3* deleted intervals (*apoa5*-F2-5'-ACAGTTGGAGCAAAGGCGTGAT-3' and *apoa5*-R2-5'-CTTGCTCGAAGCTGCCTTTCAG-3'; *apoc3*-exon4-F1-5'-CAAGTTCACCGGCTTCTGGGATT-3' and *apoc3*-exon4-R1-5'-CAGGATGGAGGAACAGGCACATC-3').

### **Double Transgenic Mouse Generation**

Human bacterial artificial chromosome (BAC) RPCI-11-442E11 DNA corresponding to the human clone publicly sequenced from this region (Genbank accession number AC007707) was prepared by standard alkaline lysis with a chromatography column (Qiagen, Valencia, CA), adjusted to a final concentration of ~1 ng/ml and micro-injected into fertilized FVB inbred



mouse eggs using standard procedures (20). Two founder transgenic mice were identified as determined by PCR amplification using primers hApoAV-intrn-F1-5'-CCCGCTGCAGTCCCCAGAAT-3' and hApoAV-intrn-R1-5'-CAGGGTCGAGGGCTCTTGTCT-3'. Each founder line was expanded by breeding to isogenic FVB strain mice (The Jackson Laboratory, Bar Harbor, MN).

### Gene Expression Analysis

Animals were sacrificed and tissues harvested for total RNA isolation using the Trizol RNA reagent (Gibco-BRL, Gaithersburg, MD). Approximately 24 µg of total RNA were separated in 1.0% agarose by gel electrophoresis and the RNA was transferred to a charged nylon membrane (Ambion, Austin, TX). The RNA blots were hybridized with [ $\alpha$ - $^{32}$ P]dCTP random-primed *apoa5* or *apoc3* probes in ULTRAhyb buffer (Ambion, Austin, TX). Mouse probe templates were generated by PCR amplification of mouse BAC RPCI-23-175F2 DNA using: 1) *apoc3*-Northern-F2-5'-GGCTGGATGGACAATCACTTCAGA-3' and *apoc3*-Northern-R2-5'-CAGGATGGAGGAACAGGCACATC-3' and 2) *apoa5*-Northern-F1-5'-ACGTAAGGCGAAGGACCTGCAC-3' and *apoa5*-Northern-R1-5'-ACAGGTCGTCCAGTCGGCTCTG-3'. Human probe templates were generated by PCR amplification of human liver cDNA using: 1) APOA1-F1-5'-CAAGAACGGCGGCCAGAA-3' and APOA1-R1-5'-GCTCTCCAGCACGGGCAGCAGG-3', 2) APOC3-F1-5'-GGAGCACCGTTAAGGACAAG-3' and APOC3-R1-5'-AGGGGGGCCAGGCATGAGGT-3', 3) APOA4-F1-5'-TGAAGGAGGAGATTGGGAAG-3' and APOA4-R1-5'-CGTTCTCCCGCAGCACTCTC-3' and 4) APOA5-F1-5'-GGAATAGAAGGGAGAAAGAG-

3' and APOA5-R1-5'-GAGATGGGGAGAGCACACAG-3'. Filters were washed in 2X saline sodium citrate at room temperature for 20 minutes and in 0.1X SSC at 42° C for 20 minutes, followed by autoradiography visualization.

### **Plasma Apolipoprotein and Lipid Analysis**

Blood samples were collected after a 5-hour fast by retro-orbital bleeding using heparinized micro-hematocrit tubes. Total cholesterol and triglyceride concentrations were measured using enzymatic methods on a Gilford System 3500 analyzer (Gilford Instruments, Oberlin, OH) (21). Plasma levels of human apo C-III were measured by kinetic immunonephelometric system (Image, Beckman Coulter) using polyclonal antibodies produced with total synthetic apoCIII in goats. Plasma levels of human apoAV were quantitated by sandwich enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies generated against a human apoAV synthetic peptide. The variation coefficient were below 6% for both methods including within and between day variation. Plasma lipoproteins from pooled mouse plasma were separated by gel filtration chromatography using a Superose 6HR 10/30 column (Pharmacia LKB Biotechnology). The gel was equilibrated with PBS (10mmol/L) containing 0.1 g/L sodium azide and plasma were eluted with the buffer at room temperature at a flow rate of 0.2 ml/min. Elution profiles were monitored at 280 nm and record with an analog-recorder chart tracing system (Pharmacia LKB biotechnology). The elution fraction numbers (0.24 ml for each) of the plasma lipoproteins separated by FPLC were VLDL, 10-18; IDL/LDL, 20-30; and HDL, 30-40. Lipids and apolipoproteins in the recovered fractions were assayed as described above.

## RESULTS

### Generation of *APOA5/APOC3* "Double Knockout" Mice

To address whether apoav and apociii exert their effect on plasma triglycerides through either a common or independent mechanism, we deleted both mouse genes and examined their resulting plasma triglyceride concentrations. Since both *apoa5* and *apoc3* are located within ~35kb of each other on mouse chromosome 9, and are thus "genetically linked", it was not feasible to directly inter-cross each of the previously generated single knockout mice to create "double knockout" animals (Figure 1). Rather, we chose to sequentially target each apolipoprotein gene in embryonic stem (ES) cells through a double-selectable marker scheme. We first disrupted the *apoa5* gene with a hygromycin-based targeting vector and subsequently deleted the *apoc3* gene with a neomycin-based targeting vector. We used properly targeted ES cell clones to generate chimeric mice and subsequently inter-crossed heterozygous double-targeted animals to generate "double knockout" mice. We obtained viable offspring at the expected Mendelian genotype ratio of 1:2:1 (15:23:15) and no obvious differences were noted in their outward physical appearance. To confirm proper targeting of the two genes, we determined the RNA levels for *apoa5* and *apoc3* from liver tissue (Figure 2A). Northern analysis revealed that "double knockout" mice completely lacked detectable *apoa5* and *apoc3* transcripts in comparisons to wild-type controls, supporting the proper targeting of the two genes.

### *APOA5/APOC3* "Double Knockouts" Display Intermediate Plasma Triglyceride Concentrations

We next examined plasma lipid levels in these genetically engineered mice. For both triglycerides and cholesterol, we found no significant differences between “double knockouts” and wild-type littermates. Plasma triglyceride concentrations were  $53 \pm 20$  mg/dL and  $44 \pm 14$  mg/dL for wild-type controls and “double knockouts”, respectively (Figure 2B) (student t-test  $p=0.4$ ). These triglyceride levels in the “double knockouts” are intermediate to those previously reported for either single *apoA5* (400%) or *apoc3* (70%) knockout mice compared to controls (2, 12). No significant difference was found in cholesterol concentrations (Figure 2B). Finally, we characterized plasma lipoprotein particles by fast protein liquid chromatography (FPLC) to determine if there was dramatic repartitioning of lipoprotein particles despite the fact that there were no significant differences in plasma lipid concentrations. Overall, we found the FPLC profiles quite similar between the controls and “double knockouts”, with no obvious differences in the various plasma particle sizes (Figure 2C).

### ***APOA5/APOC3* "Double Transgenics" Display Intermediate Plasma Triglyceride Concentrations**

To further support whether apoAV and apoCIII exert their effect on plasma triglycerides through either a common or independent mechanism, we over-expressed both human genes in transgenic mice and examined their resulting plasma triglyceride concentrations. Our first model was obtained by inter-crossing previously established human *APOA5* and *APOC3* transgenic mice (2, 13). We found that these “double transgenic” mice had intermediate plasma triglyceride and

cholesterol concentrations compared to that found in single *APOC3* and *APOA5* transgenics (Figure 3).

One potential complicating factor in our analysis of the above described “double transgenic” mice was the variable copy number and genome integration sites for each of the independently generated *APOA5* and *APOC3* transgenic lines. To reduce the possibility of artifact due to this scenario, we generated a second line of “double transgenic” mice using a different experimental design. Specifically, we selected a human bacterial artificial chromosome (BAC) containing both the *APOA5* and *APOC3* genes, as well as two interspersed apolipoprotein genes (*APOA1* and *APOA4*) (Figure 1). Our rationale for this approach was to control for identical *APOA5* and *APOC3* copy number and integration events which was not be possible through the generation of independent *APOA5* and *APOC3* transgenes. In addition, while all the genes in this cluster have been well-studied *in vivo*, only the *APOA5* and *APOC3* genes affect triglyceride concentrations, thus over-expression of *APOA1* or *APOA4* was unlikely to confound our interpretation of triglyceride concentrations in these mice. Finally, this strategy was expected to better mimic the physiologic expression of *APOA5* and *APOC3* since several regulatory elements are embedded within the larger gene cluster (Figure 1).

We generated two independent founder lines of human BAC transgenic mice and determined that all four genes (*APOA5*, *APOC3*, *APOA4*, and *APOA1*) expressed in a pattern consistent with their endogenous levels in liver and intestines as determined by Northern blot analysis (Figure 4A). Predominant expression was found in liver tissue for *APOA5*, *APOC3* and *APOA1*, and in the intestines for *APOA4*. Examination of plasma lipid concentrations revealed no differences in

triglycerides between the two transgenic lines and controls (Figure 4B). These levels in the “double transgenics” are intermediate to those previously reported for either single *apoa5* (30%) or *apoc3* (200-2000%) transgenic mice compared to controls (2, 13). For one of two double transgenic lines a slight increase in cholesterol concentrations was noted (student t-test  $p=0.016$ ) (Figure 4B). This effect might be explained by the *APOA1* expression in this model since it has been well established that *APOA1* or *APOA4* over-expression increase cholesterol concentrations and protect against atherosclerosis (5, 22). FPLC analysis indicated the lack of repartitioning of lipoprotein particles between transgenics and controls, with subtle differences found in the lipid concentrations from the peak elution volume (Figure 4C).

### **Vast Differences in ApoAV and ApoCIII Plasma Protein Concentrations in “Double Transgenic” Mice**

To determine the relative plasma levels of human apoAV and apoCIII proteins in the “double transgenic” mice, we performed immunoassays. Since our BAC “double transgenics” have identical copy number and integration sites, we chose to examine these animals for plasma protein levels. We found that ApoAV and ApoCIII levels were ~500-fold different, with double transgenics having ~0.027 mg/dL of ApoAV and ~13 mg/dL of ApoCIII (Table 1).

## DISCUSSION

The profound effect of altering ApoAV or ApoCIII levels on triglyceride concentrations in single transgenic or knockout mice previously established an important role for both proteins in triglyceride metabolism (2, 12, 13). However, their effect on triglycerides are opposite. Mice transgenic for either *APOA5* or *APOC3* have decreased and increased triglycerides, respectively; while mice inactivated for either *apo5* or *apoc3* have increased and decreased triglycerides, respectively. These findings raised the issue as to whether ApoAV and ApoCIII function in opposing roles on triglyceride homeostasis through either common or independent mechanisms. If these two proteins function in a common pathway/mechanism, one would predict that the rate limiting protein would be unaffected by alterations in the other proteins level. In contrast, if these two proteins function independently, then neither protein would be rate limiting and there would be a "tug of war" between the two processes. To address this issue, we generated "double transgenic" and "double knockout" mice for *APOA5* and *APOC3*. Our analyses of the various engineered animals revealed they all have intermediate triglyceride concentrations compared to either single gene transgenic or knockout. These data support the hypothesis that ApoAV and ApoCIII function on plasma triglycerides in an independent but opposing manner.

In both humans and mice, *APOC3* is expressed predominantly in the liver and intestines while *APOA5* expression is limited to the liver. The ApoCIII and ApoAV proteins are both found in plasma with ApoCIII localized primarily to chylomicrons and VLDL particles and ApoAV mostly to HDL particles. While it remains unclear how the newly identified ApoAV protein affects plasma triglycerides, the mechanism of action of ApoCIII has been reported. Lipid

metabolism studies in *APOC3* transgenic and knockout lines of mice indicate that these triglyceride changes are due to alterations in triglyceride catabolism by lipoprotein lipase and by chylomicron remnant clearance from the liver (12, 13, 23).

While the mechanism by which ApoAV acts on triglycerides remains to be determined, our study reveals that ApoAV is a potent triglyceride regulator relative to ApoCIII. The finding of a ~500-fold difference in ApoAV and ApoCIII plasma levels in “double transgenic” mice and yet triglyceride levels are intermediate compared to either transgenic alone suggests significant activity of small amounts of ApoAV. The fact that both *APOA5* and *APOC3* are contained in a 1:1 ratio as a component of the single transgenic fragment indicate that these protein level differences are not be due to differences in copy number or position effects. It is worth noting that the level of each plasma protein in these transgenic mice is similar to that found in humans (24-26). Previous data indicate that human ApoCIII levels range from 10-50 mg/dL which is similar to the 15 mg/dL found in our transgenic mice (24-26). Furthermore, we find human ApoAV levels are ~0.005-0.05 mg/dL (manuscript in preparation), similar to the ~0.025 mg/dL found in our double transgenics. Thus, these animals may serve as a useful model for studying these human plasma apolipoproteins in their physiological concentrations within an animal model. Subsequent interbreeding of these transgenics to “double *apoa5/apoc3* knockouts” could also provide a “humanized mouse” with respect to these two apolipoprotein genes and would provide a system to study human gene regulation such as in response to pharmacological agents.

An important clinical implication of our findings of the independent activity of ApoAV and ApoCIII relates to human genetic association studies between *APOA5* and *APOC3*



polymorphisms and triglyceride concentrations. For both genes, numerous common variants have been identified that are strongly associated with triglyceride concentrations (2, 8-11, 14-16, 18, 19, 27-30). However, the close neighboring relationship of these two genes, separated by only ~35 kb, have precluded the determination of whether both genes have functional variants, or whether polymorphisms in one of the genes are in significant linkage disequilibrium with functional polymorphisms in the other gene. While either is a possibility, the precise molecular mechanism behind this effect could ultimately be due to a single apolipoprotein (ApoAV or ApoCIII). Our mouse data support that ApoAV and ApoCIII function in an independent manner and suggest that common human variation in both genes can genetically and mechanistically contribute to triglyceride alterations. Future detailed haplotype analysis and association studies with polymorphisms throughout this cluster in a wide-range of populations should reveal the ultimate functional variants in this interval and how they relate to triglyceride metabolism.

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**TABLE 1**

	<b>Line 1</b> (n=8)	<b>Line 2</b> (n=6)
<b>ApoCIII</b> (mg/dL)	15.5±4.5	11.3±2.6
<b>ApoAV</b> (mg/dL)	2.6X10 <sup>-2</sup> ±0.007	2.7X10 <sup>-2</sup> ±0.005

**Table 1:** Human ApoAV and ApoCIII protein levels in plasma from “double transgenic” mice.



## FIGURE LEGENDS

**FIGURE 1:** Genomic organization of the human apolipoprotein gene cluster on chromosome 11q23. Genes are depicted by horizontal arrows above the schematic with known gene regulatory elements indicated with vertical arrows. The size of each gene and the distance between genes can be found below and above the figure, respectively. The identical gene order and organization is found in the orthologous mouse genomic interval, with small differences in the gene sizes and intergenic distances.

**FIGURE 2:** Analysis of *apoA5/apoC3* “double knockouts”. **(A)** Northern blot analysis of liver RNA from wild-type (WT) and double targeted (dKO) mice. **(B)** Lipid analysis of plasma from wild-type (WT) and double targeted (dKO) mice. Error bars correspond to the standard deviation for both graphs. **(C)** Plasma lipoprotein particle characterization by fast protein liquid chromatography (FPLC).

**FIGURE 3:** Plasma lipid analysis of *APOA5* transgenics, *APOC3* transgenics and *APOA5/APOC3* “double transgenics”. Double transgenic mice were generated through the intercrossing of previously established single *APOA5* and *APOC3* transgenic mice. Error bars correspond to the standard deviation for both graphs.

**FIGURE 4:** Analysis of *APOA5/APOC3/APOA4/APOA1* transgenics. **(A)** Northern blot analysis of liver and intestines RNA from wild-type (WT) and transgenic (Tg) mice. **(B)** Lipid analysis of plasma from wild-type (WT) and transgenics (Tg) mice. Two independent founder

lines were established and analyzed. Error bars correspond to the standard deviation for both graphs. Within each graph, pair-wise comparisons between controls and transgenics were insignificant (student t-test  $p > 0.05$ ), except for control versus transgenic line 2 cholesterol level comparisons ( $p = 0.016$ ) (C) Plasma lipoprotein particle characterization by fast protein liquid chromatography (FPLC).



FIGURE 2

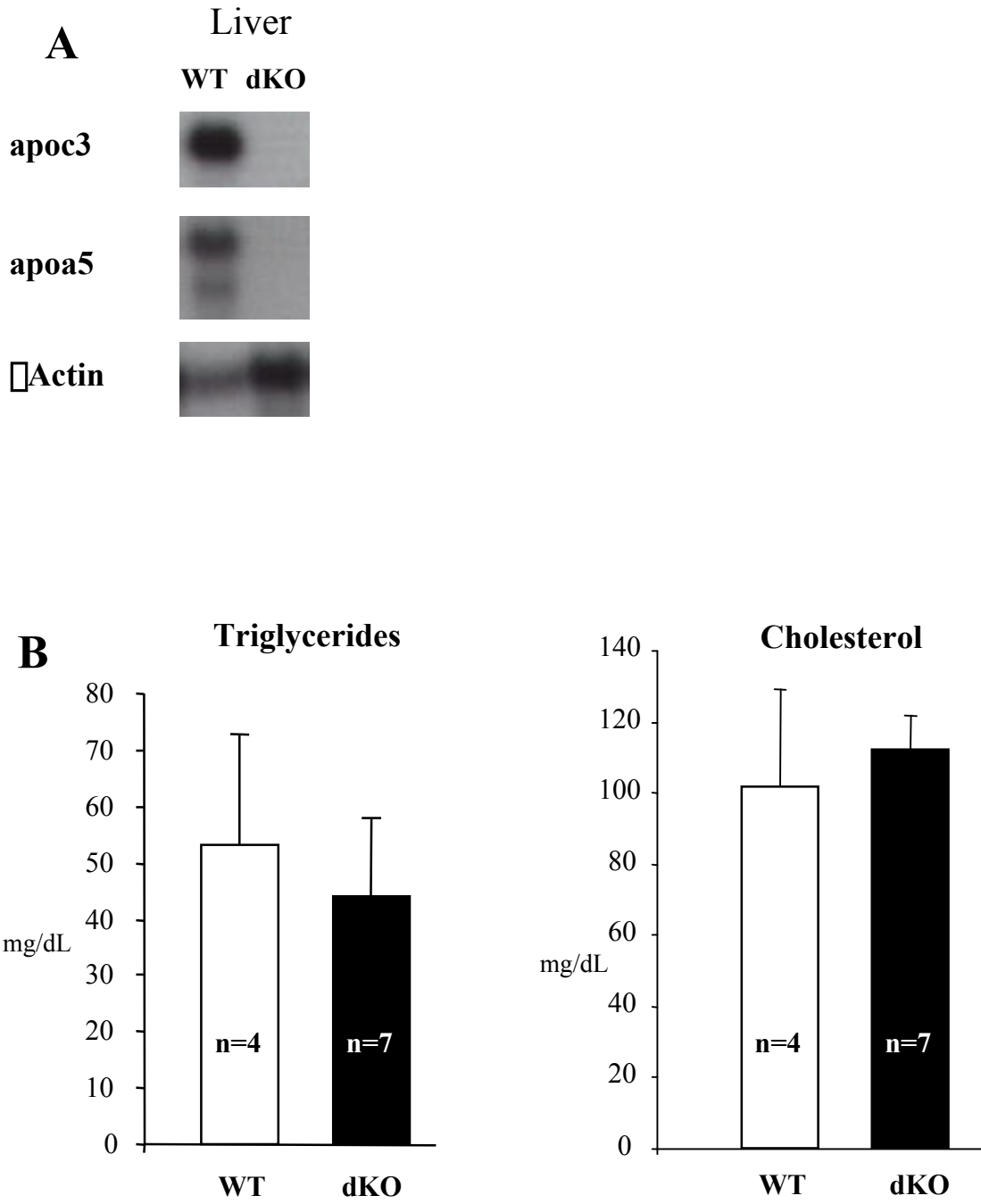


FIGURE 2 continued

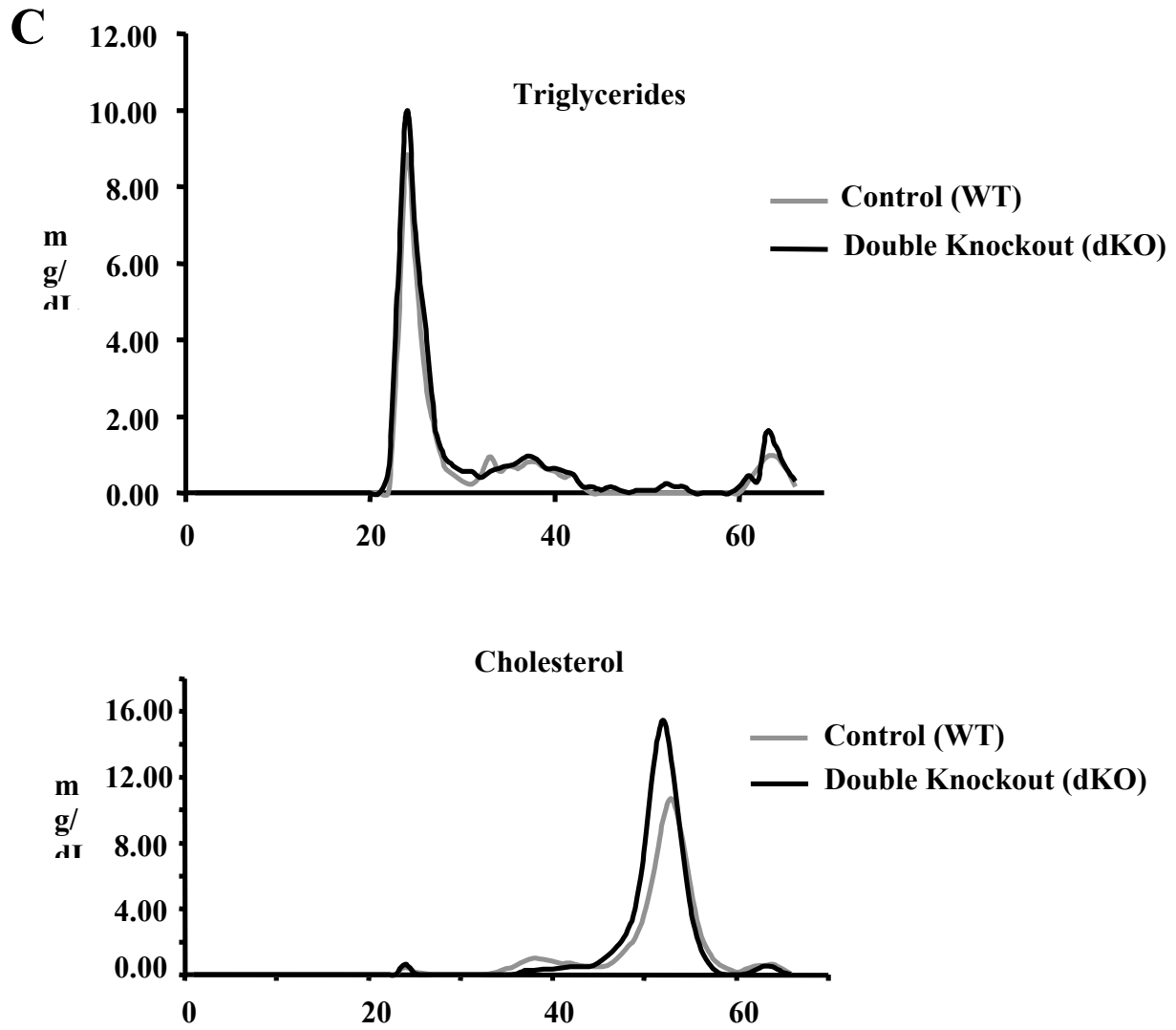


FIGURE 3

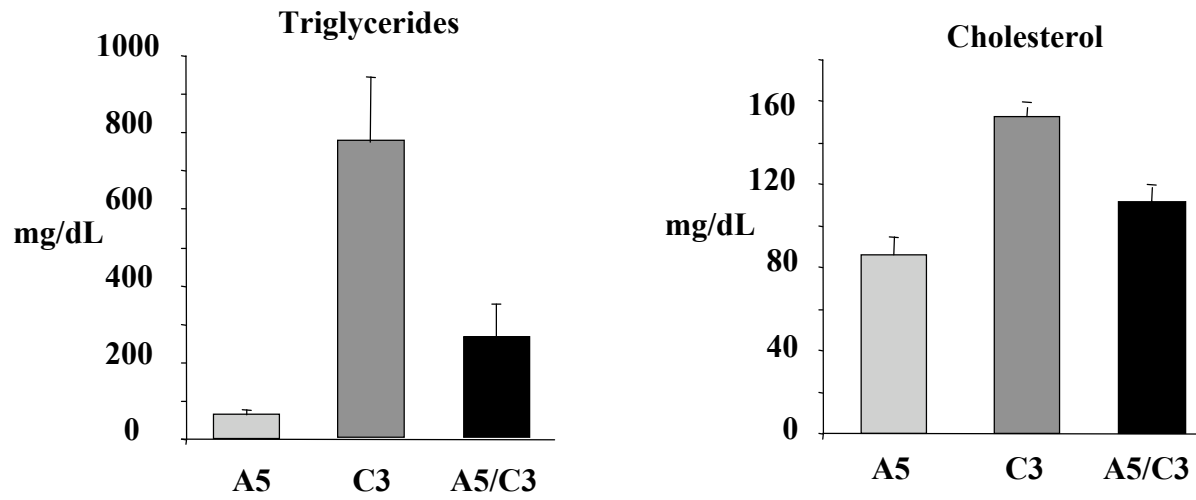


FIGURE 4

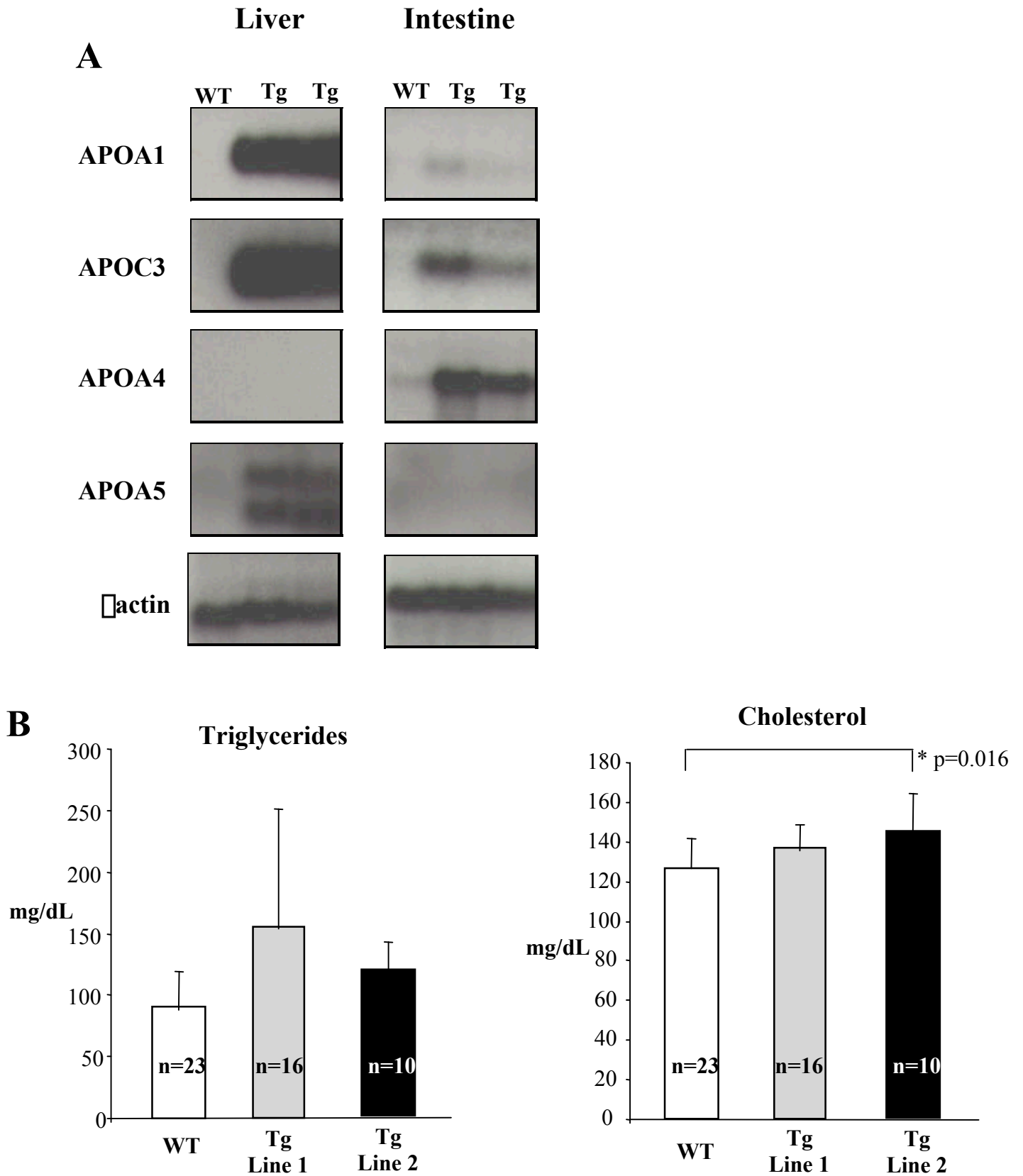


FIGURE 4 continued

