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**Article**

# Combined application of rapamycin and atorvastatin improves lipid metabolism in apolipoprotein E-deficient mice with chronic kidney disease

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**Running Title:** RAPA plus ATV reduces cardiovascular risk in CKD

**Keywords:** Chronic kidney disease, Atherosclerosis, Rapamycin, Atorvastatin, Co-administration

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*These authors contributed equally to the work.

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## ABSTRACT

Atherosclerosis arising from the pro-inflammatory conditions associated with chronic kidney disease (CKD) increases major cardiovascular morbidity and mortality. Rapamycin (RAPA) is known to inhibit atherosclerosis under CKD and non-CKD conditions, but it can cause dyslipidemia; thus, the co-application of lipid-lowering agents is recommended. Atorvastatin (ATV) has been widely used to reduce serum lipids levels, but its synergistic effect with RAPA in CKD remains unclear. Here, we analyzed the effect of their combined treatment on atherosclerosis stimulated by CKD in apolipoprotein E-deficient (*ApoE*$^{-/-}$) mice. Oil Red O staining revealed that treatment with RAPA and RAPA+ATV, but not ATV alone, significantly decreased the atherosclerotic lesions in the aorta and aortic sinus, compared to those seen in the control (CKD) group. The co-administration of RAPA and ATV improved the serum lipid profile and raised the expression levels of proteins involved in reverse cholesterol transport (LXR*α*, CYP7A1, ABCG1, PPAR*γ,* ApoA1) in the liver. The CKD group showed increased levels of various genes encoding atherosclerosis-promoting cytokines in the spleen (*Tnf-α*, *Il-6* and *Il-1β*) and aorta (*Tnf-α* and *Il-4*), and these increases were attenuated by RAPA treatment. ATV and RAPA+ATV decreased the levels of *Tnf-α* and *Il-1β* in the spleen, but not in the aorta. Together, these results indicate that, in CKD-induced *ApoE*$^{-/-}$ mice, RAPA significantly reduces the development of atherosclerosis by regulating the expression of inflammatory cytokines and the co-application of ATV improves lipid metabolism.


## INTRODUCTION

Atherosclerosis and arterial calcification are more frequent and severe in patients with chronic kidney disease (CKD) than in the general population (1). Uremia in CKD patients



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precipitates oxidative stress and inflammation in the arteries and stimulates plaque formation, in a process that is called accelerated atherosclerosis (2, 3). As a result, CKD patients exhibit increased morbidity and mortality due to cardiovascular diseases (4).

The pathogenic mechanism of atherosclerosis in CKD can be explained by an imbalance of electrolytes, such as calcium (Ca) and phosphate (P), and a loss of vascular smooth-muscle cell (VSMC) function (5). Because the kidney is the main organ for cytokine removal, CKD patients exhibit cytokine dysregulation and persistent inflammation, which can stimulate vascular cell senescence (6-8). CKD also promotes dyslipidemia; this is an alteration of cholesterol homeostasis that includes increased low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C) levels, and is known to exacerbate atherosclerosis (9).

Several studies have reported that rapamycin (RAPA) suppresses the development of atherosclerosis and arterial calcification (10, 11). However, RAPA also has been associated with hyperlipidemia in renal transplant recipients and CKD patients, because mTOR inhibition reduces the plasma lipid clearance by inhibiting the activity of lipases required for the catabolism of circulating lipoproteins and altering the expression of enzymes for fatty-acid uptake and storage (12, 13). Thus, it is recommended that lipid-lowering agents be administered together (14). The statin, atorvastatin (ATV), has been widely used to prevent major cardiovascular complications associated with hypercholesterolemia and dyslipidemia. ATV reportedly plays an anti-inflammatory role by inhibiting the production of tumor necrosis factor (TNF)-α and protecting VSMCs against TGF-β1-mediated simulation, and thereby protects against atherogenesis (15, 16). Although ATV shows a protective effect against cardiovascular disease, its use in CKD patients has been limited to date (17). A previous study found that ATV significantly reduced total cholesterol levels and LDL-C

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independent of CKD, and decreased triglyceride levels more in patients with CKD than in those without CKD (18). However, the potential synergism of RAPA and ATV (RAPA+ATV) co-treatment has not been studied in terms of atherosclerosis inhibition, especially in CKD.

To understand the mechanism of CKD-accelerated atherosclerosis in depth, *in vivo* CKD models are required. Although several mouse models have been developed for investigating the mechanisms of atherosclerosis caused by CKD, limitations exist because the genetic manipulations or inducing methods are associated with various degrees of renal failure (19). In this study, we established a distinct CKD mouse model promoting atherosclerosis compared with sham operated mice. And we investigated the effects of RAPA+ATV on the regulation of inflammatory cytokines and dyslipidemia to prevent the development of atherosclerosis in CKD-induced apolipoprotein E-deficient (*ApoE*$^{-/-}$) mice.

## RESULTS

### *RAPA ameliorates CKD-associated atherosclerosis*

To establish a consistent CKD mouse model, we used a two-step surgical nephrectomy in 8-week-old female *ApoE*$^{-/-}$ mice (Supplemental Fig. 1A). We first evaluated the serum chemistry (Table 1) and found that the levels of blood urea nitrogen (BUN), creatinine, and calcium were markedly increased in the sera of mice subjected to surgical nephrectomy compared to sera in sham-operated mice. However, RAPA and/or ATV did not decrease the uremia and the hypercalcemia associated with CKD in mice. No significant between-group difference was seen in the serum phosphate level.

To find out the effect of RAPA and/or ATV on atherosclerotic lesions accelerated by CKD, we established the CKD mouse model as described above, and the mice were further fed a Western diet to induce atherosclerosis. This model was used in all subsequent experiments.

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Oil red O staining showed that the CKD group exhibited more atherosclerotic plaque formation in the whole aorta than did the Sham group (Fig. 1A). The RAPA and RAPA+ATV groups exhibited significant reductions of the atherosclerotic lesions, whereas the ATV group did not differ from the CKD group. There was no significant difference between the RAPA and RAPA+ATV groups in this parameter. We observed similar results when we analyzed the stained aortic sinuses of mouse hearts (Fig. 1B). Together, these findings indicate that RAPA reduces the atherosclerosis associated with CKD in this model, but ATV has no additional effect on this parameter, alone or in combination with RAPA.

***Combination of RAPA and ATV improves lipid metabolism in CKD mice***

It has been reported that CKD patients may exhibit dyslipidemia, which is a major risk factor for the development of cardiovascular disease (20). To find the serum lipid levels in our experimental system, we fasted animals for 4 hours, sacrificed them, and collected whole blood for serum chemistry. The serum levels of total cholesterol and triglyceride were not different between the groups (Figs. 2A and 2B). Circulating LDL-C was significantly more increased in the CKD group than in the Sham and was inhibited in the ATV group. However, neither RAPA nor RAPA+ATV affected the LDL-C level elevated by CKD in those groups. The serum level of HDL-C in the CKD and RAPA group was similar to that in the Sham group, but the level was markedly elevated in the ATV and RAPA+ATV groups (Figs. 2C and 2D). Surprisingly, the HDL-C levels in the co-administration of RAPA and ATV were higher than that seen in the ATV group, with an increase that was twice those seen in the Sham and CKD groups. To explore the effect of RAPA and ATV on lipid metabolism, we used qRT-PCR to evaluate the mRNA expression levels of genes related to cholesterol metabolism, including *Hmgcr*, *Lxrα*, *Abcg5*, *Cyp7a1, Pparγ*, and *Apoa1*, in the livers of our experimental

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and control mice (Supplemental Figs. 2A-2F). As expected, CKD increased the mRNA expression level of *Hmgcr*, and this change was inhibited by treatment with RAPA and/or ATV. The *Hmgcr* expression level did not differ between the RAPA, ATV, and RAPA+ATV groups, and the levels of all three groups were lower than that of the sham group. CKD tended to decrease the gene expression levels of *Lxrα* compared to those in the sham group. The gene expression of *Lxrα* was significantly higher in the RAPA and/or ATV groups than in the CKD and sham groups. The classical pathway of bile acid initiated by *Cyp7a1* was more inhibited in the CKD group than in the sham group. The RAPA+ATV group had significantly higher *Cyp7a1* expression than did the CKD group. In addition, combined administration of RAPA and ATV significantly increased the mRNA level of *Abcg5*, *Pparγ* and *Apoa1,* whereas this parameter did not differ between the RAPA, ATV, sham, and CKD groups.

We subsequently examined the expression level of proteins related to reverse cholesterol transport, including LXRα, CYP7A1, ABCG1, PPARγ, and ApoA1, in the mouse liver (Figs. 3E and 3F). The CKD group significantly reduced the expression of LXRα to be similar to that in the Sham group by ATV administration, but the levels were not affected by RAPA treatment. Interestingly, the RAPA+ATV group had significantly elevated expression levels of LXRα, CYP7A1, ABCG1 and ApoA1 in the liver. The expression level of PPARγ was slightly lowered in the CKD group, but there was no significant difference between any of the groups. Given the results, we found that co-administration of RAPA and ATV is more effective in stimulating reverse cholesterol transport and bile secretion than is ATV treatment alone. These data suggest that combining RAPA with ATV may help to mitigate dyslipidemia in CKD.

***ApoE$^{-/-}$ mice with CKD exhibit up-regulation of pro-inflammatory cytokine genes in the***



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***spleen and aorta, and these levels are reduced by RAPA***

Next, we used qRT-PCR to investigate the effects of RAPA and/or ATV on the expression levels of atherogenesis-related inflammatory cytokines. In the spleen, the mRNA expression of inflammatory cytokines is known to promote atherosclerosis, including *Tnf-α*, *Il-6,* and *Il-1β*, which were specifically more increased in the CKD group than in the sham group (Figs. 4A-4C). The CKD-related up-regulations of *Tnf-α* and *Il-1β* were suppressed in the ATV, RAPA, and RAPA+ATV groups, and their levels did not significantly differ between these groups. The administration of RAPA significantly inhibited the CKD-related increase of *Il-6* expression, whereas this level was similar among the ATV, ATV+RAPA, and CKD groups. Interestingly, *Il-10*, an anti-inflammatory cytokine, was more increased in the CKD group than in the sham group, and this increase was suppressed in the ATV and RAPA+ATV groups (Fig. 4D).

In the aorta, the mRNA levels of *Tnf-α* and *Il-4* were higher in the CKD group than in the sham group (Figs. 4E and 4F), whereas the RAPA group had significantly lower levels of *Tnf-α* and *Il-4* than did the CKD group. However, ATV and RAPA+ATV did not decrease their levels like RAPA did. Surprisingly, the mRNA expression level of *Il-6* did not differ in any of the groups (Fig. 4G). The mRNA level of *Il-10* was significantly increased in the RAPA group over that in the sham and CKD groups (Fig. 4H), but this significant increase was not seen in the RAPA+ATV group. These results suggest that the administration of RAPA helps decrease the levels of atherosclerosis-promoting cytokines and increases those of atherosclerosis-suppressing cytokines, but that combined treatment with RAPA and ATV does not appear to show these synergistic effects.

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## DISCUSSION

We herein provide the first report on how combined treatment with RAPA and ATV affects the atherogenesis stimulated by CKD. In our study, the oral administration of RAPA in CKD-induced *ApoE*$^{-/-}$ mice ameliorated atherosclerotic lesions and inhibited the mRNA expression levels of pro-inflammatory cytokines in the spleen and aorta. These data confirm that RAPA plays an athero-protective role by reducing the pro-inflammatory burden in CKD. Although ATV did not additionally reduce the aortic lesions, the combined use of ATV markedly increased the serum levels of HDL-C in *ApoE*$^{-/-}$ mice with CDK. Thus, our results suggest that the combination therapy of RAPA plus ATV helps to alleviate the atheroprone environment of CKD.

The formation of atherosclerotic lesions can differ with the stage of renal failure. An electronic cautery-based renal injury mouse model has been used in various CKD studies, because such mice exhibit substantial increases in their serum levels of BUN and creatinine (21, 22). However, these methods did not show sufficient or consistent renal impairment in our preliminary experiments (data not shown). We instead used high-temperature battery-operated cautery to forcefully damage the kidney and found that this procedure yielded a consistent CKD model. The serum calcium and phosphate levels of operated mice were significantly elevated, supporting the idea that our model is suitable for the mechanistic study of CKD. Since uncontrolled lipid homeostasis promotes atherosclerosis, the ability of the reverse cholesterol-transport pathway to eliminate excessive serum lipids is an important factor in reducing the risk of cardiovascular disease (23). Previous study revealed that experimental chronic uremia increased serum LCL-C and stimulated atherosclerosis in mice (24). Likewise, we found more LDL-C in the serum of CKD-induced mice than in the Sham. However, there were no differences in the serum total cholesterol and triglyceride between

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the two groups. These findings suggest that maintaining the balance of LCL-C and HDL-C is important for improving atherosclerosis in CKD.

RAPA has been shown to significantly suppress atherosclerosis in studies using animal models with normal renal function (10, 11), but there are still concerns about adverse effects that may be associated with RAPA treatment, such as dyslipidemia. In our results, atherosclerotic lesions were substantially reduced by RAPA administration in CKD mice, but the serum lipids levels were not influenced. Several studies have reported that mTOR inhibition limits the serum lipid elimination by suppressing lipase activity and cholesterol trafficking (12, 13, 25, 26). It was also reported that RAPA treatment increases circulating PCSK9 levels, which are related to the increased serum LDL-C and hypercholesterolemia in patients with nephrotic syndrome (27, 28). These findings support the limitation of RAPA use in CKD patients. Further study will be needed to elucidate the role of RAPA in PSCK9-associated dyslipidemia. Several researchers suggested that combined treatment with a statin would reduce the possibility of RAPA-induced dyslipidemia, resulting in additional amelioration of atherosclerosis (29, 30). This prompted us to examine the potential of ATV to counter this disadvantage of RAPA treatment. Consistent with previous findings, our results showed that the decreased expression of LXRα and CYP7A1 in the liver of CKD mice was compensated by ATV administration. LXRα has long been suggested as a therapeutic target against atherosclerosis, since it is implicated in cholesterol efflux and hepatic bile-acid synthesis by regulating the expression of target genes associated with reverse cholesterol transport, including CYP7A1, ABCG1/5/8 and apolipoproteins (31, 32). Meanwhile, LXRα increases fatty-acid (FA) and triglyceride (TG) synthesis by upregulating genes, including SREBPc, FA synthase, and acetyl coenzyme A carboxylase (33). However, accumulated work has clearly shown the beneficial effect of LXRα agonist against atherosclerosis. Furthermore,

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213 our study demonstrated that CKD mice treated with RAPA plus ATV gained weight
214 (Supplementary Fig. 1B) and exhibited stabilization of the HDL-C level (Fig. 2C). The
215 combined use of RAPA and ATV further promoted the expression of ABCG1 and ApoA1,
216 which contribute more to cholesterol efflux than does RAPA or ATV alone. Given that
217 hepatic ApoA1 synthesis decreases and HDL-C level falls are common effects of renal failure
218 (34), our study suggests that the activation of LXRα/ApoA1/ABCG1 by RAPA and ATV co-
219 administration improves the stability of HDL-C and atherosclerosis in CKD (35). These
220 results suggest that the combined treatment could be synergistic in eliminating the excessive
221 serum lipids promoting atherosclerosis, and improving general conditions and long-term
222 mortality in mice with renal failure.

223 Dysregulation of cytokines in CKD is associated with a significant decrease in cytokine
224 secretion, given that the kidney is a main organ for eliminating cytokines (9). RAPA was
225 previously shown to target pro-inflammatory cytokines and mTOR activation induced by
226 chronic consumption of a high-fat diet (36). Indeed, we found that RAPA, ATV, and
227 RAPA+ATV all markedly decreased the gene expression levels of atherosclerosis-promoting
228 inflammatory cytokines such as *Tnf-α, Il-6* and *Il-1β* in the spleen. Interestingly, the mRNA
229 level of the anti-inflammatory cytokine, *Il-10*, was increased in the spleen tissues of the CKD
230 group. This appears to be a compensatory mechanism intended to correct inflammation in
231 CKD (38). In the aorta, RAPA specifically decreased the level of *Il-4* and increased the level
232 of *Il-10*, indicating that it exerts anti-inflammatory effects to prevent atherogenesis.
233 Conversely, these levels were similar in the ATV, RAPA+ATV, and CKD groups. Several
234 reports have suggested that ATV can have inhibitory effects on atherosclerosis in non-CKD
235 models (39, 40). Thus, the previous and present results demonstrate that RAPA and ATV can
236 both regulate the systemic inflammation environment in CKD patients, but that these effects

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are not synergistic.

In conclusion, we herein show that RAPA can play a critical role in reducing the development of atherosclerosis in the aortas of CKD-induced *ApoE*$^{-/-}$ mice by alleviating systemic inflammation. Although the co-administration of ATV did not further reduce atherosclerosis, it improved the lipid profile and bile-acid metabolism in these mice. Thus, the combined administration of RAPA and ATV can provide synergistic effects in alleviating the cardiovascular risk associated with CKD, beyond the effects of either agent alone.

## MATERIALS AND METHODS

The detailed methods are described in the "Supplementary Information".

## ACKNOWLEDGMENTS

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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## FIGURE LEGENDS

Table 1. Body weight and laboratory data of *ApoE*$^{-/-}$ mice with CKD at the end of the study

Figure 1. RAPA ameliorates the formation of atherosclerotic plaques, as assessed by Oil red O staining. (A) Representative *en face* images of whole aortas (left) and quantification of lesion areas from the indicated groups (right) (*ApoE*$^{-/-}$; $n$ = 6-7 per group). (B) Representative images of frozen sections of aortic sinuses (left) and quantification of plaque areas on aortic sinuses of the indicated groups (right) ($n$ = 3-5 per group). Data are shown as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the CKD group.

Figure 2. Combined treatment with RAPA plus ATV has beneficial effects on lipid metabolism. Serum lipid profiles for (A) total cholesterol, (B) triglycerides, (C) low-density lipoprotein cholesterol (LDL-C), and (D) high-density lipoprotein cholesterol (HDL-C) from the indicated groups ($n$ = 6-9). (E, F) The expression levels of reverse cholesterol transport-related proteins in the livers of the indicated groups ($n$ = 5 per group). Data are shown as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the CKD group.

Figure 3. The effects of RAPA and ATV on the CKD-related stimulation of pro-atherosclerosis cytokines in *ApoE*$^{-/-}$ mice. Gene expression levels of inflammatory cytokines in the spleen (A-D) and aorta (E-H) ($n$ = 5-7 per group). Data are shown as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the CKD group.

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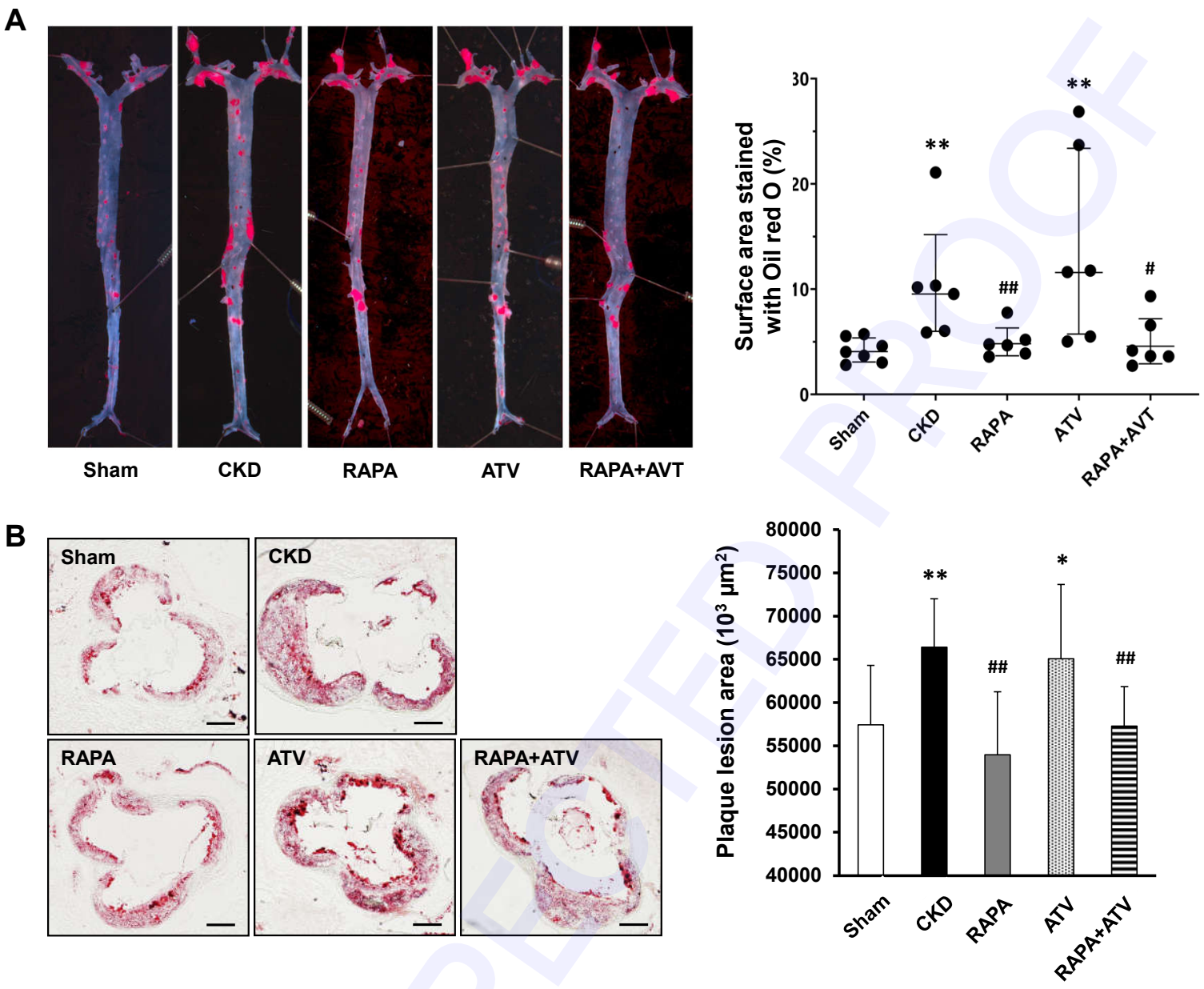
# Figure 1

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# Figure 2

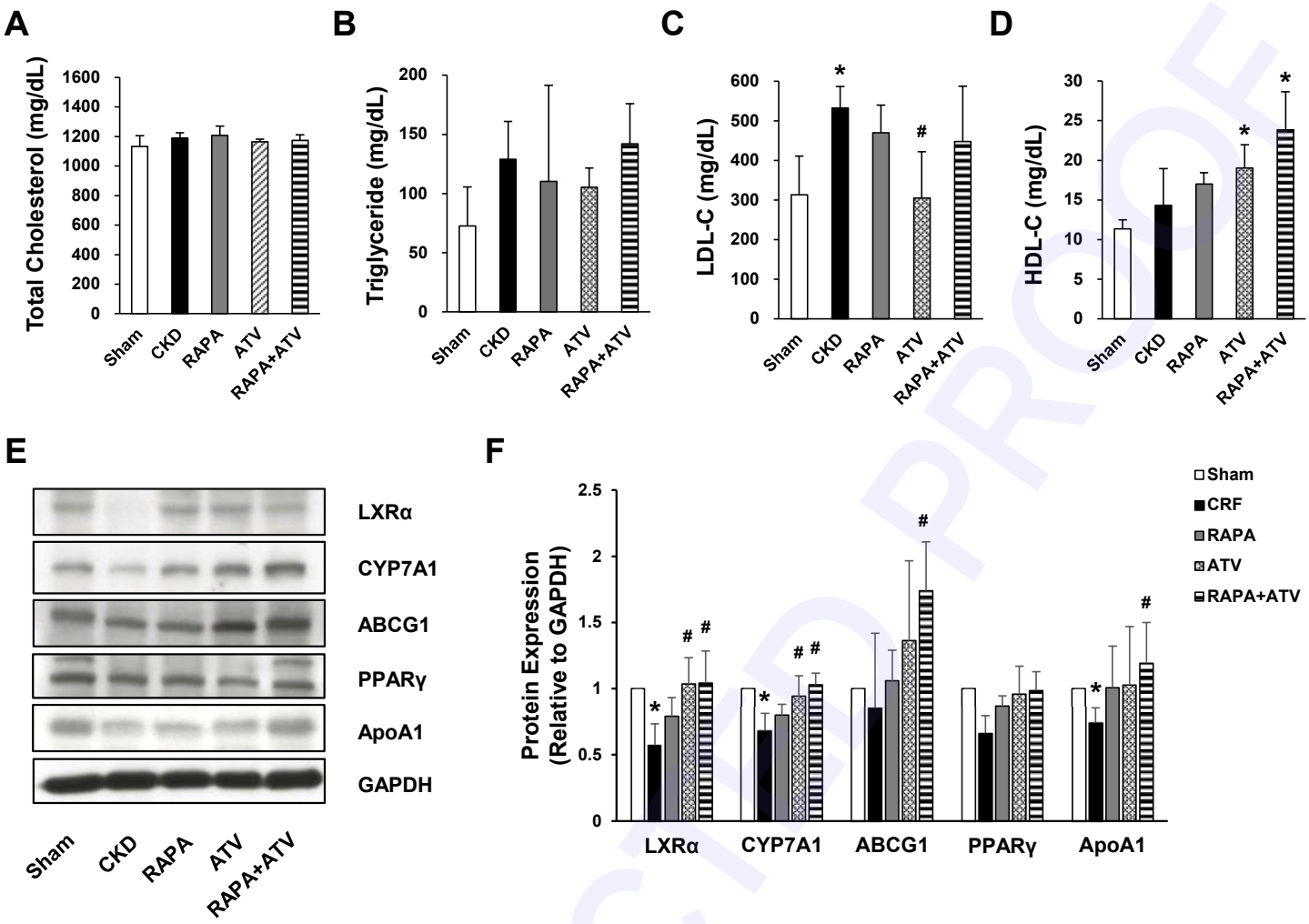





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**Figure 3**

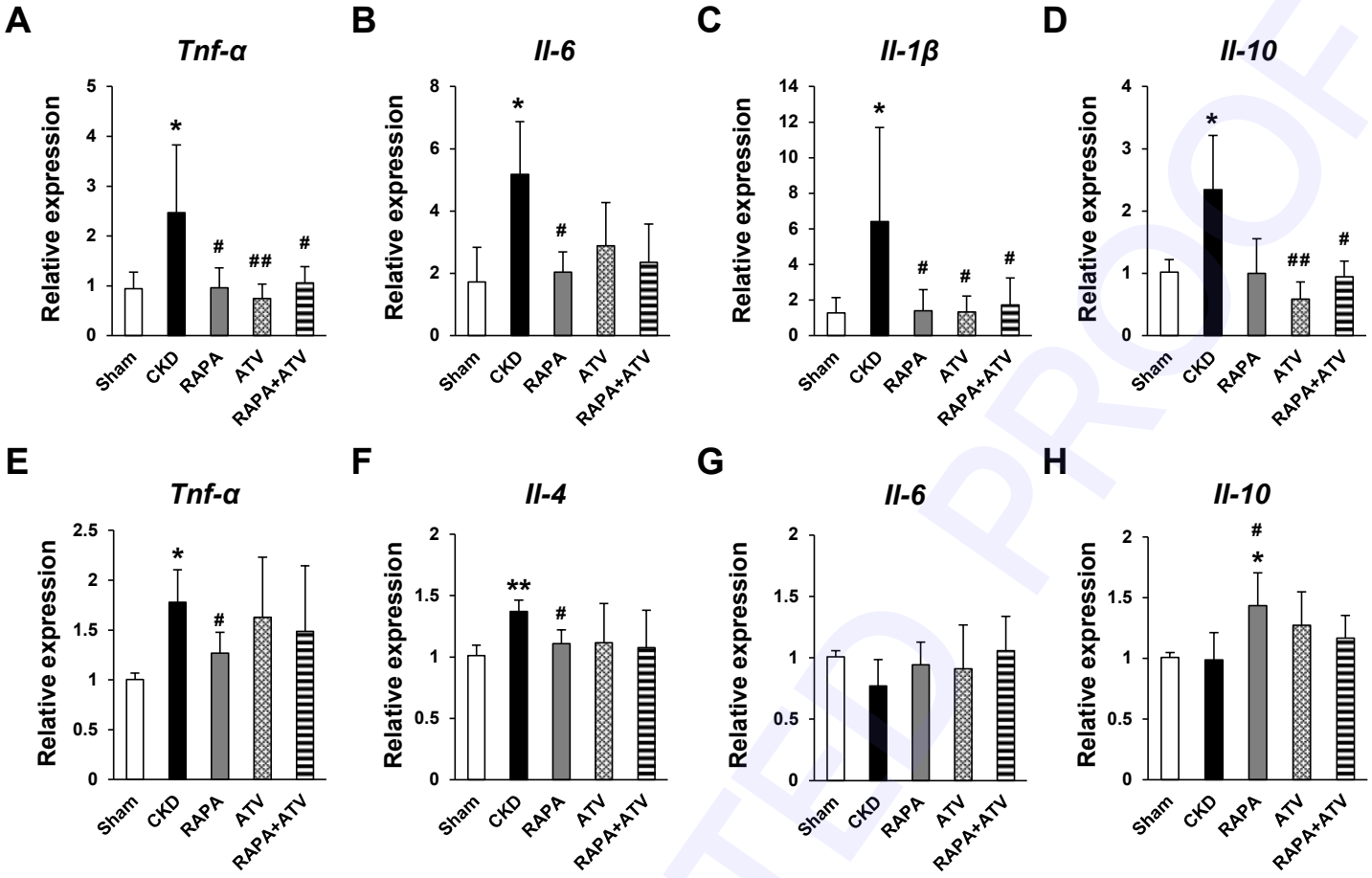


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**Table 1. Body weight and laboratory data of *ApoE*$^{-/-}$ mice with CKD at the end of the study**

| | Sham | CKD | RAPA | ATV | RAPA+ATV |
|---|---|---|---|---|---|
| **Body weight (g)** | 22.9 ± 1.2 | *20.4 ± 2.4 | *20.3 ± 3.0 | *20.1 ± 4.1 | #22.7 ± 1.5 |
| **BUN, mg/dL** | 18.7 ± 3.9 | *92.8 ± 14.4 | *78.9 ± 32.8 | *79.5 ± 19.5 | *65.8 ± 25.2 |
| **Creatinine, mg/dL** | 0.28 ± 0.02 | *0.47 ± 0.03 | *0.65 ± 0.13 | *0.59 ± 0.19 | *0.56 ± 0.14 |
| **B/C ratio** | 44.4 ± 39.8 | *195.3 ± 19.2 | *111.8 ± 28.9 | *128.8 ± 24.3 | *151.3 ± 23.3 |
| **Calcium, mg/dL** | 9.78 ± 0.39 | *11.27 ± 1.48 | *10.10 ± 0.67 | *10.17 ± 0.7 | *11.08 ± 0.42 |
| **Phosphate, mg/dL** | 7.23 ± 0.79 | 8.0 ± 2.0 | 6.57 ± 1.78 | 6.73 ± 1.08 | 8.72 ± 1 |
| **Total protein, g/dL** | 6.13 ± 1.4 | 8.27 ± 3.33 | 7.94 ± 2.86 | 4.45 ± 0.31 | 9.21 ± 4.76 |
| **Albumin, g/dL** | 1.67 ± 0.21 | 1.77 ± 0.4 | 1.81 ± 0.39 | 1.28 ± 0.05 | 1.99 ± 0.62 |

BUN, blood urea nitrogen; B/C, BUN/Creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.
Data are shown as mean ± SEM, *$P < 0.05$, **$P < 0.01$ compared with Sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with CKD group.

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**Article**

# Combined application of rapamycin and atorvastatin improves lipid metabolism in apolipoprotein E-deficient mice with chronic kidney disease

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**Running Title:** RAPA plus ATV reduces cardiovascular risk in CKD

**Keywords:** Chronic kidney disease, Atherosclerosis, Rapamycin, Atorvastatin, Co-administration

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**#**These authors contributed equally to the work.

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## MATERIALS AND METHODS

### *Experimentally induced CKD mouse model (two-step procedure)*

All animal studies were approved by the Institutional Animal Care and Use Committee of Ewha Womans University (IACUC-18-036). All experiments were carried out on female *ApoE*$^{-/-}$ mice at 8 weeks of age. The animals were maintained in a specific pathogen-free facility set on a 12-h light-dark cycle, and were given free access to chow diets and sterilized water. A two-step procedure was used to induce uremia. Briefly, the right kidney was dissected from the adrenal gland and surrounding fat through a 2-cm flank incision; it was then cauterized by Bovie High-Temperature Battery-Operated Cautery (Symmetry Surgical, TN, USA), except for 2 mm around the hilum. Two weeks later, the ureter and renal artery and vein were clipped using a surgical clip and left total nephrectomy was performed through a left flank incision. The sham operation used as a control comprised the decapsulation of both kidneys. After 2 weeks of recovery, the mice were divided into the CKD, RAPA, ATV, and RAPA+ATV groups and fed a Western diet (#D12097B; Research Diets, Inc., NJ, USA) for 10 weeks to induce atherogenesis. Where indicated, rapamycin (RAPA; 0.5 mg/kg) or/and atorvastatin (ATV; 10 mg/kg) were given by oral gavage for 5 days a week for 10 weeks. Rapamycin and atorvastatin were supplied by Pfizer.

### *Blood biochemistry*

Whole blood was drawn through the retro-orbital plexus of each deeply anesthetized mouse using a heparin-treated capillary. Serum was separated by centrifugation at 1,500 g for 15 min and stored at -80 ℃ until analysis. Serum levels of BUN, creatinine, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein

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cholesterol (LDL-C) were measured using a Hitachi 7180 biochemistry autoanalyzer (Hitachi Ltd., Tokyo, Japan). The levels of calcium, phosphate, total protein, and albumin were also measured.

### *Atherosclerotic lesion analysis*

Mice were euthanized with carbon dioxide inhalation, and the hearts and aortas were perfused through the left ventricle with ice-cold phosphate-buffered saline (PBS). Hearts, including the aortic roots, were embedded in frozen-section compound (3801480; Leica, IL, USA) and serially sectioned at 7 μm. For *en face* analysis, the aorta was opened along the longitudinal axis and pinned onto a black wax plate. For measurement of atherosclerotic plaque lesions, the aorta and the heart section were fixed with 10% formalin in PBS and stained using an Oil red O solution. The lesion areas were analyzed using the Axio Vision software (Carl Zeiss, Jena, Germany).

### *RNA isolation and quantitative real-time PCR*

Total RNA from tissue samples was prepared with the TRIzol reagent (Gibco, CA, USA), and cDNA was synthesized with the Maxime$^{TM}$ RT-PCR PreMix (iNtRON Biotechnology, Korea). Quantitative real-time PCR (SYBR® FAST, Kappa Biosystems, MA, USA) was conducted to determine the relative levels of mRNA using a 7700 sequence detector (Applied Biosystems, CA, USA) and primers for the target mouse genes (Supplemenatry Table 1). The data were normalized to the mRNA level of *Gapdh* in each reaction.

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### *Western Blot Analysis*

Total protein was extracted from tissue samples using EzRIPA Lysis buffer containing protease and phosphatase inhibitor cocktail (ATTO, Tokyo, Japan). For analysis of target protein expression, proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was incubated in primary antibodies at 4 ℃ for overnight, and with secondary antibodies for 2 hours at room temperature. Immunoreactive band were visualized and quantified. Antibodies for target protein were purchased as followed: anti-LXRα (Abcam, Cambridge, UK); anti-Cyp7a1, anti-PPARγ, anti-IL6 and anti-VCAM1 (Santa Cruz Biotechnology, CA, USA); anti-ABCG1 (Novus Biologicals, CO, USA); anti-ApoA1 (Biodesign, ME, USA); anti-GAPDH and HRP-conjugated goat anti-mouse/rabbit IgG antibodies (GeneTex, TX, USA).

### *Statistical analysis*

Continuous variables are expressed as the mean ± standard deviation and were compared using the Mann-Whiney *U* test. Categorical variables were tested with Fisher's exact tests and are expressed as counts and percentages. Body weights were analyzed using a repeated-measures ANOVA followed by Bonferroni's post-hoc test. Analyses were performed with the SPSS Statistical Analytics software (IBM Analytics, NY, USA).

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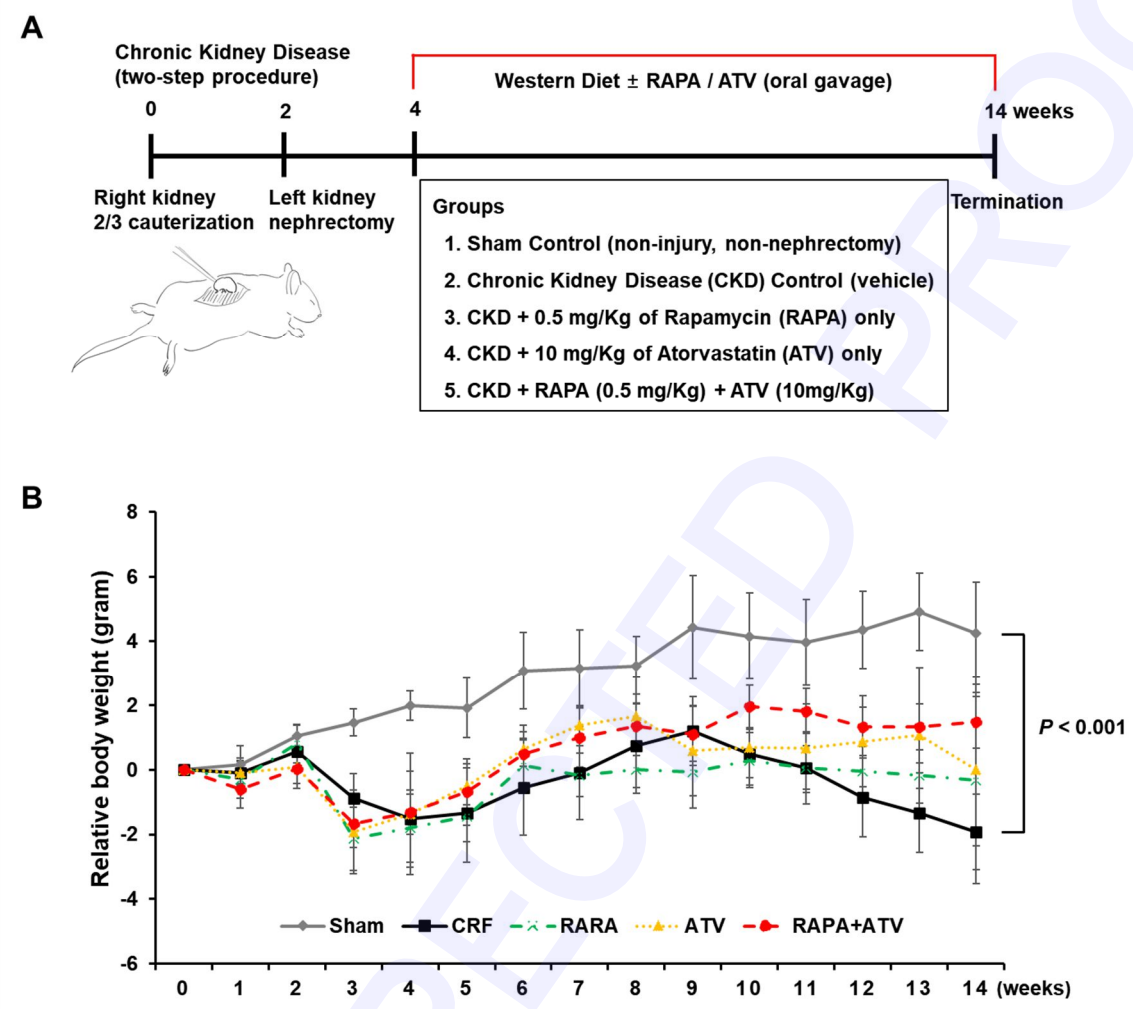
## SUPPLEMENTARY FIGURES




Supplementary Figure 1. (A) Schematic presentation of the experimental protocol. (B) Changes in the body weights of experimental animals.

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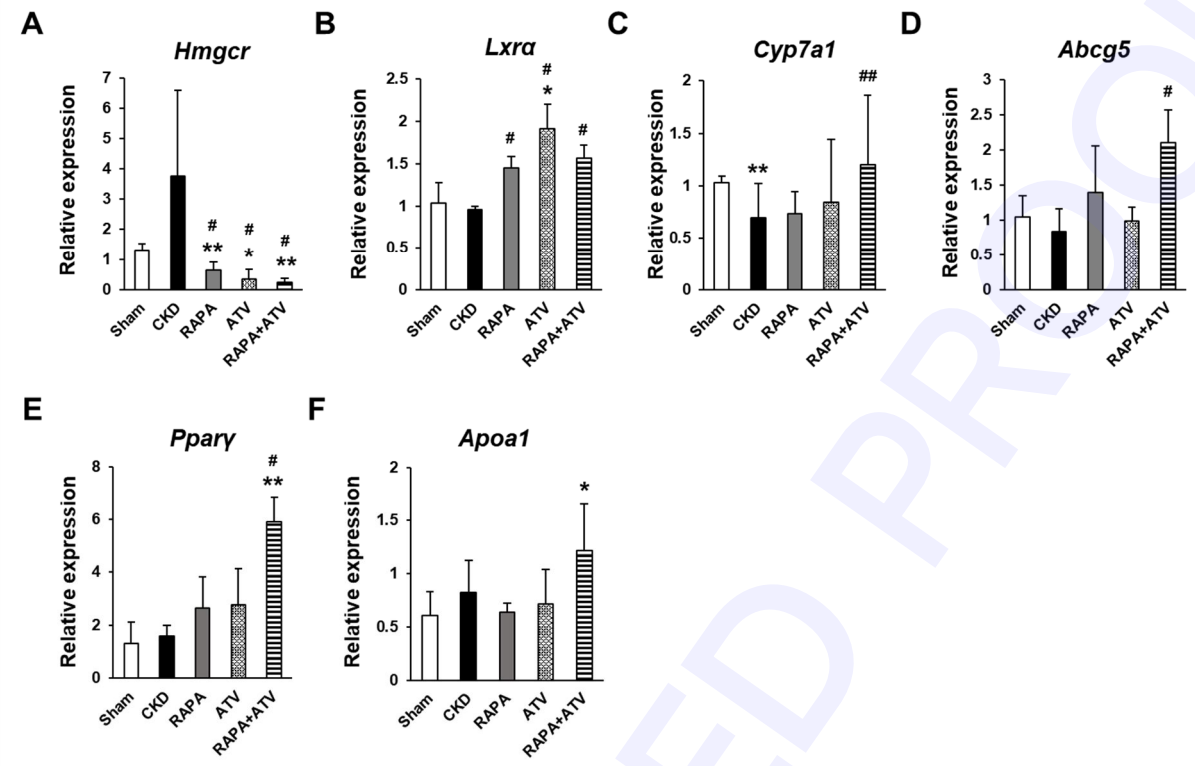


Supplementary Figure 2. mRNA expression levels of (A) cholesterol metabolism-related genes, (B, C) cholesterol transport-related genes, (D) bile acid biosynthesis genes, and (E, F) lipid and glucose metabolism-related genes in the livers of the indicated groups (n=5-7 per group). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the CKD group.

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## SUPPLEMENTARY TABLE

Supplementary Table 1. Primer sequences

| | Sequence (5' -> 3') | |
|---|---|---|
| **Gene** | **Forward primer** | **Reverse primer** |
| *Hmgcr* | TTTCTAGAGCGAGTGCATTAGCA | GATTGCCATTCCACGAGCTATAT-3’ |
| *Lxrα* | GATGTTTCTCCTGATTCTGCAAC | AGGACTTGAGGAGGTGAGGAC |
| *Abcg5* | CCTGCTGAGGCGAGTAACAA | TGGCACCCACAAGCTGATAG |
| *Cyp7a1* | CACTCTACACCTTGAGGATGG | GACATATTGTAGCTCCTGATCC |
| *Pparγ* | AGATTCAGAAGAAGAACCGGAAC | CCGATCTCCACAGCAAATTATAG |
| *Apoa1* | GCATGCGCACACACGTAGACTCTCT | CGTCTCCAGCATGGGCATCAGACTA |
| *Tnf-α* | TGGCCCAGACCCTCACACTCAG | ACCCATCGGCTGGCACCACT |
| *Il-6* | CTTCCATCCAGTTGCCTTCTTG | AATTAAGCCTCCGACTTGTGAAG |
| *Il-1β* | GGAGAACCAAGCAACACAAAATA | TGGGGAACTCTGCAGACTCAAAC |
| *Il-10* | GCTCTTACTGACTGGCATGAG | CGCAGCTCTAGGAGCATGTG |
| *Il-4* | GAATGTACCAGGAGCCATATC | CTCAGTACTACGATGAATCCA |
| *Gapdh* | CATCACTGCCACCCAGAAGACTG | ATGCCAGTGAGCTTCCCGTTCAG |

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