

# Angiotensin-converting Enzyme Inhibitors Have Adverse Effects in Anti-angiogenesis Therapy for Hepatocellular Carcinoma

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## Research

**Keywords:** Hepatocellular carcinoma, Anti-angiogenic drugs, Proteinuria, ACEIs, Erythropoietin

**Posted Date:** September 10th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-72739/v1>

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**Version of Record:** A version of this preprint was published on March 31st, 2021. See the published version at <https://doi.org/10.1016/j.canlet.2020.12.031>.

# Abstract

**Background:** Currently, most clinicians prescribe angiotensin-converting enzyme inhibitors (ACEIs) to alleviate proteinuria caused by anti-angiogenic drugs (AADs) according to diabetic nephropathy guidelines or expert recommendations. However, the efficacy ACEIs and potential cancer promoting effect are controversial. This study aimed at confirming whether ACEIs are beneficial in anti-angiogenesis therapy for hepatocellular carcinoma.

**Methods:** In clinic, we observed the impact of ACEIs on AADs during the treatment of hepatocellular carcinoma. Next, we established different tumor-bearing mouse models to confirm that whether ACEIs have any effect on the anti-cancer efficacy and proteinuria side effects of AADs. Further, we confirmed the relevant mechanism in vivo.

**Results:** Our clinical data have shown that some hepatocellular carcinoma (HCC) patients experienced tumor progression by ACEIs administration for the treatment of hypertension or proteinuria caused by AADs. We have confirmed that in different tumor-bearing mouse models, ACEIs did not delay the appearance of proteinuria or alleviate proteinuria caused by AADs but compromised the anticancer efficacy of AADs. This effect is unrelated to a change in the VEGF signaling pathway. Our data showed that the combination of ACEIs and AADs flared the production of kidney-derived erythropoietin (EPO). In turn, EPO compromises the anti-angiogenic effects of AADs and decreases antitumor activity.

**Conclusions:** For the treatment of proteinuria caused by AADs, ACEIs have no efficacy but promote drug resistance. Kidney-derived EPO is mainly responsible for ACEIs induced anti-angiogenesis resistance.

## Background

Approximately half of patients with HCC have reached an advanced stage, and in these patients, systemic drug therapy is the most important option for prolonging survival. HCC is a typical blood vessel-rich tumor, and anti-angiogenesis-related therapies have become the standard treatment for patients with advanced HCC[1-3]. To date, the US Food and Drug Administration has approved 4 oral tyrosine kinase inhibitors (TKIs) and 1 anti-angiogenic antibody for the treatment of advanced HCC[4]. Meanwhile, the combined application of immune checkpoint inhibitors and TKIs is the current clinical research trend, and the results are promising[5, 6]. Whether used alone or in combination with other drugs, AADs will become the main choice for systemic treatment of HCC in the near future.

AADs improve the overall survival of patients with HCC, but their side effects may require the administered dose to be reduced, thereby limiting the clinical benefit[7]. The three side effects of the most concern are hand-foot syndrome, hypertension and proteinuria[8]. Except for proteinuria, hand-foot syndrome and hypertension can be well controlled, and good management strategies for these side effects have been developed according to previous clinical experience. It has been reported that AAD-related proteinuria occurs in 30% to 70% of patients[9-11]. Many patients undergo dose reduction or temporary stop use of AADs, which in turn leads to compromised therapeutic effects. Therefore, good

management of AAD-related proteinuria plays a vital role in ensuring the efficacy of anti-angiogenic therapy and the stable development of new therapies targeting this pathway[8]. However, regarding tumor anti-angiogenic therapy, nephrotoxicity associated with pharmacological inhibition of VEGF signaling in the kidney and its downstream pathways remains a concern. Although the side effects of AAD-related proteinuria have been well described in numerous experimental and clinical studies, to date, no strategy has been established to reduce proteinuria[12-14].

Currently, most clinicians prescribe ACEIs to treat proteinuria caused by AADs according to diabetic nephropathy guidelines, expert recommendations or drug instructions to reduce the occurrence or degree of proteinuria. However, there is no definitive evidence showing that ACEIs reduce the proteinuria caused by AADs. Moreover, it remains controversial whether ACEIs promote cancer. Some studies show that the use of ACEIs is closely related to tumor progression and a low survival rate. However, most studies have relatively small sample sizes and short follow-up times, and some are designed to assess overall cancer risk but not to specifically assess the risk of a certain type of cancer[15-17]. Hicks et al. conducted a large cohort study including one million people and confirmed the use of ACEIs associated with increased overall lung cancer risk[18]. Wysocki et al. found that captopril promotes the growth of immunogenic tumors and promotes tumor recurrence after surgery in a mouse model of renal cell carcinoma[19]. Emile et al. found that ACEIs reduce the efficacy of bevacizumab in the treatment of ovarian cancer[20]. Based on these results, we question whether ACEIs are suitable for the treatment of AAD-related proteinuria.

Nakamura et al. confirmed that hypoxia in the kidneys induced by AADs would increase circulating EPO levels[21]. Some studies have shown that EPO protects tumor blood vessels from damage by AADs and thereby reduces its antitumor effect, blocking EPO function can inhibit tumor angiogenesis and significantly increase the antitumor activity of AADs[21, 22]. Pradeep et al. discovered another EPO receptor, erythropoietin HCC type B receptor 4 (EphB4). It can trigger downstream signaling and promote EPO-induced tumor growth and progression[23]. The above studies reveal that EPO is the key to weakening the anticancer effect of AADs. The renin-angiotensin system (RAS) is an endocrine system of peripheral blood and has been established as a key regulator of blood pressure and steady-state volume. Some studies have proven that angiotensin II has a close relationship with EPO[24, 25].

Here, we demonstrate using two different tumor-bearing mouse models that ACEIs do not interfere with the effect of AADs on the VEGF signaling pathway or with the anti-angiogenic effects of the kidney, which indicates that ACEIs have no efficacy for delaying or reducing proteinuria caused by AADs. We further show that ACEIs compromise the anticancer efficacy of AADs without affecting the VEGF signaling pathway. Finally, we provide evidence that ACEIs promote AAD-induced renal EPO production, which may be a cause of AADs resistance.

## Materials And Methods

### Patient data

Patient information were obtained from the follow-up data of the patients enrolled in the clinical trials (Study of Apatinib in Advanced Hepatocellular Carcinoma) we applied. Patient AFP, proteinuria and imaging data are all available in our hospital medical record system. Details of the clinical trial are available at <https://clinicaltrials.gov/>, identifier NCT03046979.

### **Cell lines and transfections**

MHCC-97H and Hep1-6 cells were donated by the Liver Cancer Institute of Zhong Shan Hospital in Shanghai and maintained in Dulbecco's modified Eagle's medium (DMEM) (Corning 10-013-CV, USA) supplemented with 10% fetal bovine serum (FBS) (Corning 35-056-CM, USA), 10 U/mL penicillin (Corning 30-001-CI, USA), and 10 mg/mL streptomycin (Corning 61-088-RM, USA). The mycoplasma test was performed with a Mycoplasma Detection Kit, and short tandem repeat (STR) analysis was conducted for cell line authentication. All cells were maintained at 37°C in 5% CO<sub>2</sub>. Luciferase lentivirus was produced by Shanghai Ji Kai Gene Chemical Technology Co., Ltd., and the viral vector was confirmed by DNA sequencing. MHCC-97H cells were prepared by stable infection with luciferase lentivirus and selected with puromycin following the instructions.

### **Establishment of a tumor-bearing mouse model and treatment with ACEIs and AADs**

Four-week-old male BALB/c nude mice and C57BL/6 male mice were bred, acclimated, and caged in groups of six or fewer per cage at the Experimental Animal Center of Tianjin Medical University Cancer Institute and Hospital under specific pathogen-free conditions. MHCC-97H cells ( $1 \times 10^7$ ) or MHCC-97H cells transfected with luciferase lentivirus ( $1 \times 10^6$ ) suspended in 100  $\mu$ L PBS were injected subcutaneously into the lateral forearm of nude mice, and Hep1-6 cells ( $1 \times 10^7$ ) were injected subcutaneously into C57BL/6 mice in the same way. Subcutaneous tumors were allowed to establish for approximately 1 week or 2 weeks. Once the tumor was established, the mice were randomly grouped. Mice were then treated with ACEIs (captopril, enalapril or fosinopril) or AADs (apatinib or regofenib) once per day by oral gavage. Apatinib was diluted in 0.5% (w/v) carboxymethyl cellulose and 5% (w/v) glucose solution, and ACEIs were soluble in the same solvent. Regorafenib was dissolved in transcutol/cremophor/sodium chloride (1:1:8), and ACEIs were soluble in the same solvent. Tumor volume was monitored three times a week. Tumor volumes were estimated by measuring the largest diameter (A), the corresponding perpendicular (B), and the perpendicular to the plane formed by A and B (C), according to the formula  $(A \times B \times C)/2$ . The curves of tumor growth was drawn according to tumor volume and the time of implantation. The time to death of mice in each group was recorded to plot survival curves.

### **Tumor tissues, blood and organ collection**

Mice were killed after the experimental observations. Necropsy was performed, the tumor tissues, kidneys, liver and lungs were removed, and the quality of the kidneys and tumor were measured. A fraction of the tissues and organs was immediately fixed with 4% (wt/vol) paraformaldehyde (PFA) overnight, followed

by washing with PBS and embedding with paraffin until further use. Blood samples were collected from all animals, and serum samples were kept at  $-80^{\circ}\text{C}$  until further use.

## Urine analyses

Metabolic cages were used to collect 24-hour urine from mice one time per week. The gross albuminuria of each group was detected using Coomassie staining of 10  $\mu\text{l}$  of urine run on an SDS-PAGE gel. Quantitative analysis was measured using an ELISA kit (Bethyl, USA), and urine creatinine was measured using a colorimetric assay kit (Exocell, USA) following the manufacturer's methodology.

## Histological evaluation

Paraffin-embedded tissues were sectioned at a thickness of 3  $\mu\text{m}$  and stained with hematoxylin and eosin (H&E) or Periodic acid Schiff (PAS) and Masson's trichrome staining kits (Sigma, USA), according to standard techniques. Tissues were imaged with a universal upright fluorescence microscope and imaging system (OLYMPUS BX61, USA). The glomerular damage and liver and lung metastatic tumor nodules on each slice were evaluated and counted by an experienced pathologist, and the proportion of damaged glomeruli was calculated. The mean glomerular volume area was calculated based on the average volume of 30 glomeruli in each group, and the equation  $GV = (\beta/\kappa) \times GA^{3/2}$  (1), where  $\beta = 1.38$ , the shape coefficient of spheres (the idealized shape of glomeruli),  $\kappa = 1.1$ , the size distribution coefficient, and GA is the glomerular area[26].

## Fresh-frozen immunofluorescence staining and imaging

Frozen sections of tissues were prepared by the Department of Pathology of our hospital. Fresh-frozen tumor and kidney sections were fixed in methanol (Tianjin Fengchuan Chemical Reagent Technology Co., Ltd., China) for 20 minutes at  $-20^{\circ}\text{C}$ . Then, the sections were incubated with blocking solution containing 1% bovine serum albumin (BSA) and 5% FBS in PBS at room temperature for 3 h. Tissues were incubated overnight with primary antibodies diluted in blocking solution. We used rabbit polyclonal antibodies against CD31 (Abcam, ab28364, USA) as primary antibodies. After 3 washes for 5 min each with blocking solution, samples were incubated with secondary antibodies diluted in PBS for 1 h. The secondary antibody was goat anti-rabbit IgG H&L (Alexa Fluor® 488) preadsorbed (ab150081, USA) from Abcam. Samples were washed 3 times with PBS and subsequently stained with DAPI to reveal nuclei, which were diluted in PBS at a concentration of 1  $\mu\text{g}/\text{ml}$ . Sections were mounted in Dako Fluorescence Mounting Medium, covered with a cover glass, and stored at  $4^{\circ}\text{C}$ . Images were captured with a universal inverted fluorescence microscope (Leica DMI6000B, USA), and ImageJ software was used for image analysis and quantification.

## Western blot analyses

Tumor and kidney tissues were extracted according to the manufacturer's instructions of the ProteinExt® Mammalian Total Protein Extraction Kit (TransGen Biotech, Beijing, China). Western blotting was

performed with primary antibodies against VEGFA (Abcam, ab1316, USA), P-VEGFR2 (Abcam, ab5473, USA), VEGFR2 (Abcam, ab39256, USA),  $\beta$ -actin (Abcam, ab179467, USA), EphB4 (Abcam, ab254301, USA), EPO (Abcam, ab24202, USA), HIF-1 $\alpha$  (R&D, MAB1536, USA) and HIF-2 $\alpha$ /EPAS1 (Novus, NB100-122, USA). Mouse/rabbit secondary antibodies were from Cell Signaling Technology (#7076, #7074, USA). Protein levels were analyzed using Gel-Pro Analyzer Version 4.0 (Media Cybernetics, MD, USA).

### **Real-time PCR**

The whole operation process of real-time PCR (RT-PCR) reactions was carried out as described by Alissa C. et al[27]. The EPO primers were synthesized by Biotechnology Engineering (Shanghai, China) Co., Ltd., and the sequences were EPO-s: 5'-AATGGAGGTGGAAGAACAGGCCAT-3', EPO-as: 5'-CGAAGCAGTGAAGTGAGGCTACGTA-3'.

### **EPO ELISA**

Serum levels of mouse EPO were quantified by using an ELISA method according to the instructions (Abcam, ab119522, USA).

### **Immunohistochemistry**

EPO and VEGFA immunohistochemical staining was performed using DakoAutostainer Link 48. Slides of kidney tissues were incubated in 3% hydrogen peroxide for 10 min. Specific antibodies against EPO or VEGFA were added, and samples were incubated for 40 min at room temperature. Primary antibodies: EPO (1:50, Abcam, ab245127, USA) and VEGFA (1:200, Abcam, ab1316, USA). After rinsing with wash buffer, a mouse/rabbit linker (Santa Cruz, sc2357, USA) was applied to the tissue, and samples were incubated for 10 min. Samples were incubated with the secondary antibody for 20 min. Appropriate positive and negative controls were used with each run of immunostaining. The entire section examined was recorded by a pathologist blinded to the western blot results. Staining scale: 0 = no staining, 1 = weak, 2 = moderate, 3 = strong and proportion of the immunopositive staining area: 0= 0%-10%, 1= 10%-25%, 2= 25%-50%, 3= 50%-100%.

### **Statistical analysis**

GraphPad Prism 8.0 (GraphPad Prism Software, La Jolla, CA, USA) was used for statistical analyses. All values are expressed as the mean  $\pm$  SD. For analyses of two groups, we performed T-tests if data had normal distributions; for analyses of more than two groups, we performed ANOVA with Tukey's test for multiple comparisons or Sidak's test for comparisons against a single control group. The Kaplan–Meier method was used to analyze the survival of mice. All statistical tests were performed as two-tailed analyses, and  $P < 0.05$  was considered statistically significant.

## **Results**

## **Captopril does not delay or alleviate proteinuria caused by apatinib and may counteract the anticancer efficacy of apatinib in HCC patients and tumor-bearing mouse models**

Apatinib, a specific small molecule tyrosine kinase inhibitor (TKI), can highly selectively inhibit the tyrosine kinase activity of vascular endothelial growth factor receptor 2 (VEGFR-2). In China, apatinib has been recommended as second-line treatment of advanced gastric cancer and HCC[28-30]. From 2016 to the present, we used single-agent apatinib to treat 208 patients with advanced HCC. Apatinib exhibited a convincing anticancer effect and acceptable toxicity[31, 32]. We found that Captopril did not reduce the proteinuria caused by apatinib, and several patients even experienced deterioration. Patient 1 with severe advanced HCC received apatinib for 26 months and was in clinical, radiological and biological partial response when she developed severe hypertension and proteinuria. She was then administered captopril and experienced progression within the next 18 months. Alpha-fetoprotein (AFP) showed an upward trend throughout the entire captopril administration time and an occasional decrease because of the doubled dose of apatinib. The dose had to be reduced frequently because of intolerance. Captopril did not prevent the production of severe proteinuria caused by apatinib during the entire use process. Eventually, after the patient stopped using apatinib, the proteinuria level gradually decreased (Figure 1a). Patient 2 was also in radiological and biological partial response after 3 months of apatinib treatment. In the fourth month, she began taking captopril due to hypertension and proteinuria, and she then experienced progressive disease. After discontinuing captopril, the response to apatinib resumed (Supplemental Figure 1, a and c). The proteinuria was not alleviated by the use of captopril. Patient 3 had a partial response (PR) after 2 months of apatinib treatment. Subsequently, the patient was administered captopril at the same time due to proteinuria, and the disease progressed (Supplemental Figure 1, b).

Next, we subcutaneously injected MHCC-97H cells labeled with luciferase into the lateral forearm of BALB/c (nu/nu) male mice. Once tumors were established, the mice were given apatinib (200 mg/kg) daily, and the bioluminescence intensity of the mice was detected weekly. One week of apatinib administration decreased the luminescence signal by more than 60%, but the signal recovered after one week of supplementation with captopril (30 mg/kg/day) and decreased again after captopril was stopped for a week. The same trend was found in the next two dosing cycles (Figure 1b). These data suggest that ACEIs do not delay or reduce the proteinuria caused by AADs but rather may counteract the anti-angiogenic effect of AADs.

## **Determining the dose of drug used in the tumor-bearing mouse model and establishing a stable proteinuria model related to AADs**

Because captopril was reported to promote tumor growth in an immunogenic model and decrease the survival of immunogenic mice in a dose-dependent manner[33], we established two mouse subcutaneous tumor models, the immunogenic C57BL/6 mouse model with Hep1-6 cells and the immunodeficient BALB/c nude mouse model with MHCC-97H cells to verify this effect. To generalize the role of ACEIs, we chose three representative drugs: captopril containing thiol, enalapril without thiol and fosinopril containing phosphorus[34]. After tumors were established (Figure 2, a and e), the mice received different

doses of ACEIs [vehicle, high-dose captopril (60 mg/kg/day), low-dose captopril (30 mg/kg/day), high-dose enalapril (60 mg/kg/day), low-dose enalapril (30 mg/kg/day), high-dose fosinopril (10 mg/kg/day) and low-dose fosinopril (5 mg/kg/day)], which were based on the low and high doses used in humans according to a body surface area dose conversion method, as reported in previous literature[19, 35, 36]. The high dose was designed to be twice the low dose because the recommended dose of ACEIs for the treatment of proteinuria is twice that for the treatment of hypertension according to diabetic nephropathy guidelines[37, 38]. We observed that ACEIs decreased the survival of tumor-bearing immunogenic mice in a dose-dependent manner (Supplemental Figure 2a). However, in immunodeficient tumor-bearing mice, we found that only high-dose ACEIs reduced survival time, whereas low-dose ACEIs did not affect survival time (Supplemental Figure 2b). Regardless of immunogenic or immunodeficient tumor-bearing mice, high and low doses of ACEIs did not affect tumor growth (Supplemental Figure 2, c and d). These results prompted us to choose low-dose ACEIs in subsequent animal experiments.

The doses of apatinib in the treatment of tumor-bearing mice were 50 mg/kg/day, 100 mg/kg/day and 200 mg/kg/day according to the previous literature[39-41]. A comparison of the anticancer ability of different doses and whether various doses can lead to stable proteinuria have not been reported. Our results showed that in both liver cancer animal models, 200 mg/kg/day apatinib exhibited the best anticancer effect, prolonged the survival of tumor-bearing mice (Figure 2, b and f) and inhibited tumor growth (Figure 2, c and g). Importantly, the 200 mg/kg/day apatinib group produced stable proteinuria after two weeks (Figure 2, d and h). Targeting VEGF may cause two types of glomerular injury: thrombotic microangiopathy (TMA) and focal segmental glomerulopathy (FSGS) [42]. Both of them were observed in our apatinib-induced proteinuria mouse model (Figure 2i). These data confirmed that the optimal dose of apatinib was selected when studying the effect of apatinib on proteinuria.

### **Captopril does not reduce proteinuria or kidney damage caused by apatinib**

In clinical practice, cancer patients may use ACEIs after the diagnosis of proteinuria caused by AADs. To fit the clinical situation, we administered apatinib to tumor-bearing mice for 2 weeks in advance to ensure stable proteinuria and then administered captopril to determine whether captopril could alleviate proteinuria (Figure 3a). From the initiation of captopril administration, the 24-hour urine of mice was collected via metabolic cages each week. From the weekly dynamic quantitative test and the qualitative test of the last week of captopril administration, the administration of captopril was not found to effectively alleviate albuminuria (Figure 3, b and c). Histological analysis of HE-stained, PAS-stained and Masson-stained kidney samples showed that captopril treatment neither attenuated the degree of glomerular injury nor reduced the proportion of damaged glomeruli (Figure 3, d and e). CD31 immunofluorescence staining of the kidneys showed a significant decrease in microblood vessels after apatinib treatment, which was not attenuated by captopril treatment (Figure 3f). Furthermore, captopril did not interfere with the effect of apatinib on the molecular level of the renal VEGF signaling pathway (Figure 3g). These data confirmed that captopril cannot reduce the proteinuria or kidney damage caused by apatinib.

## **ACEIs do not delay proteinuria or kidney damage caused by AADs**

Although ACEIs have a definite effect on delaying proteinuria in diabetic nephropathy, ACEIs had no significant effect on the event of death[43]. Next, we sought to observe whether ACEIs could delay the appearance of proteinuria caused by AADs. We simultaneously administered different ACEIs and AADs to tumor-bearing mice (Figure 4a, Supplemental Figure 4a). Because Regorafenib has been shown to induce a higher rate of proteinuria when used to treat liver cancer[44], we selected apatinib and regorafenib as representatives of AADs, and we selected captopril, enalapril and fosinopril as representatives of ACEIs. Our data showed that although the combined use of ACEIs and AADs in the first three weeks resulted in lower quantified proteinuria than AADs alone, the difference was not statistically significant. After proteinuria was stabilized, proteinuria under treatment with ACEIs and AADs was almost the same as proteinuria induced by AADs alone (Figure 4b, Supplemental Figure 4c). Qualitative proteinuria in the urine of mice in the last week also confirmed that the combination of ACEIs could not reduce the proteinuria caused by AADs (Supplemental Figure 3a, Supplemental Figure 4b). Glomerular pathology analysis confirmed that the combination of ACEIs could not reduce the degree of glomerular damage or the proportion of damaged glomeruli caused by AADs (Supplemental Figure 3b, Figure 4c, Supplemental Figure 4, d and g). In view of the presence of glomeruli and renal hypertrophy in diabetic mice, we observed that neither AADs alone nor AADs combined with ACEIs caused hypertrophy or atrophy of the glomeruli and kidneys (Supplemental Figure 3, c and d, Supplemental Figure 4, e and f). Further experiments confirmed that ACEIs did not affect the inhibition of AADs on renal vascular and VEGF signaling pathways (Figure 4, d and e, Supplemental Figure 4h). Together, the above results indicated that ACEIs could not delay the occurrence of proteinuria end events caused by AADs.

## **ACEIs do not affect the reduction in proteinuria or the recovery of kidney injury after AAD withdrawal**

As shown in Figure 1a, we found that after the use of AADs was stopped in patients, proteinuria gradually decreased and disappeared. Administration of the drugs was stopped after three weeks of treatment with apatinib and captopril, and the proteinuria of the mice was tested every week after administration (Figure 5a). We found that the proteinuria of the mice gradually disappeared after three weeks of apatinib withdrawal (Figure 5b). Pathological analysis of the kidneys revealed that glomerular damage was alleviated, and the proportion of damaged glomeruli was reduced from approximately 80% to 25% (Figure 4c and Figure 5d). Together, these results suggested that ACEIs do not delay or reduce the proteinuria caused by AADs. However, proteinuria gradually disappears, and kidney damage recovers after AAD withdrawal.

## **ACEIs reduce the anticancer efficacy of AADs, and their combined treatment promotes liver and lung metastasis in tumor-bearing mice with high metastatic potential**

We tested three kinds of ACEIs and two kinds of AADs in two animal models to observe whether ACEIs could reduce the anticancer effect of AADs in different drug combinations. In tumor-bearing immunodeficient mice with MHCC-97H cells, we found that the use of low-dose ACEIs alone affected neither survival nor tumor growth. The combination of ACEIs and AADs inhibited tumor growth in tumor-

bearing mice and improved survival. However, the improvement observed for the combined use was significantly lower than that observed for the use of AADs alone (Figure 6, a, b, d and e). We also measured the volume and mass of the tumors at specific time points. The combined use of ACEIs and AADs still corresponded to significantly higher tumor volume and mass than the use of AADs alone (Figure 6, c and f). We also observed liver and lung metastasis in mice. In the combined treatment group, some mice with subcutaneous tumors were found to have metastasis to the liver and lungs, in the ACEIs alone or AADs alone group, no liver or lung metastases were observed (Figure 6g).

In tumor-bearing immunogenic mice with Hep1-6 cells, low-dose ACEIs did not affect tumor growth or reduce the survival time. Although the combined use of ACEIs and AADs improved survival and inhibited tumor growth, the effects were significantly lower than those of AADs alone (Supplemental Figure 5). Consistent with the clinical observations shown in Figure 1 and Supplemental Figure 1, ACEIs did have an effect on reducing the anticancer efficacy of AADs, and the combined treatment of ACEIs and AADs may also promote the metastasis of cancer cells.

### **ACEIs reduce the anticancer efficacy of AADs by promoting the expression of kidney-derived EPO**

In the process of exploring the mechanism by which ACEIs reduce the anticancer efficacy of AADs, a phenomenon that has attracted our attention, we noticed that the capillaries on the surface of the subcutaneous tumor were almost invisible in the nude mice treated with apatinib. However, after combined treatment with captopril, the capillaries on the surface of the tumor reappeared (Supplemental Figure 6a). We doubt whether the combined use of ACEIs reduces the anti-angiogenic efficacy of AADs. CD31 immunofluorescence staining revealed that, unlike in the kidney, ACEIs reduced the anti-angiogenesis effect of AADs in the tumor mass (Figure 7a, Supplemental Figure 6b). We further explored the effect on the VEGF signaling pathway in the tumor mass. As in the kidney, ACEIs did not interfere with the inhibition of VEGF signaling by AADs (Figure 7b, Supplemental Figure 6c). Because activation of the EPO signaling pathway has been well proven to be a main reason for the off-target anti-angiogenesis effect of AADs[21, 23], we analyzed the EPO signal of tumor tissues. The EPO and EphB4 protein levels in tumor tissues of the ACEI combination group were significantly higher than those of the AADs alone group (Figure 7c, Supplemental Figure 6d). The trend of EPO levels in the mouse serum was consistent with that in the tumor tissue (Figure 7d, Supplemental Figure 6e).

The liver, kidney, spleen and tumor are potential organs or tissues for EPO synthesis[21, 27]. Therefore, EPO mRNA was measured in these tissues, and no significant change was observed in the liver, spleen or tumor tissues. However, a significant increase in EPO mRNA was detected in the kidney in the ACEI combination group (Figure 7e, Supplemental Figure 6f). Western blotting and immunohistochemical staining further confirmed that the combination of ACEIs increased the levels of protein related to the EPO pathway to a greater degree than the use of AADs alone without affecting the VEGF signaling pathway (Figure 7f and 7g, Supplemental Figure 6g). These findings indicate that ACEIs aggravate the production of renal EPO caused by AADs, which leads to the rapid emergence of AAD resistance.

## Discussion

Our data support the following main findings. (a) ACEIs cannot reduce or delay proteinuria caused by AADs, (b) ACEIs alone do not promote tumor growth of immune integrity and immunodeficiency liver cancer subcutaneous tumor mouse model. (c) High doses of apatinib easily induce stable proteinuria, but the anticancer effect of apatinib is better. (d) Proteinuria caused by AADs is reversible after withdrawal. (e) The combination of ACEIs and AADs promotes the production of kidney-derived EPO and reduces the anticancer efficacy of AADs. In summary, ACEIs cannot relieve AAD-induced proteinuria but promote drug resistance during the treatment of hepatocellular carcinoma.

Maintenance of the physiological function of the glomerular filtration barrier requires fine crosstalk between VEGF on podocytes and VEGFR2 on endothelial cells[45, 46]. VEGF levels that are too high or too low can both lead to proteinuria[47, 48]. Proteinuria caused by diabetic nephropathy has been shown to be closely related to activation of the VEGF signaling pathway, and the pharmacological inhibition of VEGF helps reduce diabetic nephropathy-related proteinuria[49]. The proteinuria caused by AADs is an effect of inhibition of the VEGF signaling pathway[8], therefore, considering the VEGF signaling pathway, the mechanism of the occurrence of proteinuria is opposite. Some researchers believe that ACEIs delay proteinuria in diabetic nephropathy due to inhibition of the VEGF signaling pathway[50]. Our results indicate that ACEIs alone or in combination with AADs do not affect the kidney's VEGF signal (Figure 4 and 5). According to our experimental results, ACEIs are unlikely to reduce proteinuria caused by AADs. As shown in Figure 4b and Supplemental Figure 4c, the combination of ACEIs at the initial stage slightly reduced proteinuria, but the difference was not significant. Rather, we speculate that the effect of ACEIs on hemodynamics reduces renal perfusion and may have a certain effect on proteinuria in the initial stage, although this effect is slight and time-limited. Unfortunately, our findings indicate that ACEIs have no efficacy in reducing or delaying proteinuria caused by AADs.

It has been reported that proteinuria caused by AADs can be restored after drug withdrawal. We verified this point in our tumor-bearing mouse model. The reduction in proteinuria is inseparable from reactivation of the renal VEGF signaling pathway, angiogenesis, repair of glomerular injury and functional recovery. However, discontinuation also means halting the anti-angiogenic therapy against the tumor. It is well believed that the counterattack of tumor angiogenesis is also rapid and that the tumor will rapidly progress after drug withdrawal. Many oncologists believe that drug withdrawal should only be implemented with caution. The optimal management strategy for increasing or decreasing AADs and drug withdrawal requires further evidence-based research. AAD-induced nephrotoxicity versus anticancer efficacy seems to be an irreconcilable paradox. However, a considerable number of patients still receive AADs in the clinic who do not develop proteinuria, which may be related to the patient's basic renal function and the tolerance of the kidneys to AADs. Further investigations are needed to determine how to reduce or reverse proteinuria without compromising the antitumor effect of AADs.

The results of epidemiological studies on the relationship between ACEIs and malignant tumors are controversial and this controversy has been driven by insufficient follow-up, regardless of cancer type and

imperfect methodology[15, 17, 51]. Higher quality research has gradually emerged in recent years. For example, Hicks et al. confirmed that the long-term use of ACEIs for more than 5 years clearly promotes lung cancer[18, 52]. The effects of ACEIs in tumor-bearing mice also differ across different tumor types, different tumor-bearing mouse models and different drug dosages. Similar to the study by Piotr J et al., we focused on the effects of well-tolerated doses of ACEIs on tumor growth in mice[19]. Our results show that regardless of immunogenic or immunodeficient tumor-bearing mice, ACEIs alone do not affect tumor growth. This result may be strongly related to the mouse model. Our research is consistent with the effect of ACEIs on subcutaneous tumors confirmed by other studies[53, 54].

It has been reported that angiotensin system inhibitors can improve vessel perfusion and promote nanomedicine or chemotherapy drug delivery, thereby improving the efficacy of apoptotic drugs[53, 54]. The effect of angiotensin system inhibitors on AADs has not been well elucidated, and here, we rule out immune interference to prove that ACEIs reduce the anticancer efficacy of AADs against liver cancer. Supplemental Figure 6a shows the conversion of AAD-treated subcutaneous tumors from white to red upon administration of ACEIs. As shown in Supplemental Figure 6f, the experimental tumors treated with ACEIs were more prone to intratumoral hemorrhage. These phenomena are all related to the fact that ACEIs promote tumor blood vessel perfusion. Importantly, CD31 staining clearly showed that ACEIs reduce the anti-angiogenic effects of AADs. Therefore, we proved that ACEIs compromise the anticancer activity of AADs.

We performed a preliminary investigation to further delineate why ACEIs reduce the anticancer efficacy of AADs. ACEIs do not affect the VEGF signaling pathway of tumors but promote the production of renal EPO caused by AADs. Renal-derived EPO may then act on the tumor site to cause AAD resistance. Because our data confirmed that physiologically tolerable doses of ACEIs are ineffective against proteinuria caused by AADs and that they promote the development of AAD resistance, we believe that the combined use of ACEIs in cancer patients who are being treated with AADs should be carefully reconsidered. Taken together, ACEIs have no efficacy for the treatment of proteinuria caused by AADs, but promote drug resistance. Kidney-derived EPO is mainly responsible for ACEIs induced anti-angiogenesis resistance. Most importantly, ACEIs should be used very cautiously in patients with anti-angiogenic therapy.

## Conclusion

Taken together, our data show that systemic anti-angiogenesis treatment leads to a reduction in tumor blood vessels and reduces kidney blood vessels, which is the root cause of kidney damage and proteinuria. ACEIs cannot reduce the damage of AADs to the kidneys but increase the production of EPO induced by AADs, which indirectly accelerates the resistance of tumors to AADs (Figure 8). This study is of great significance to guide the use of ACEIs in the process of antiangiogenic therapy in patients with hepatocellular carcinoma.

## Abbreviations

ACEIs: Angiotensin-converting enzyme inhibitors; AADs: Anti-angiogenic drugs; HCC: Hepatocellular carcinoma; EPO: Erythropoietin; TKIs: Tyrosine kinase inhibitors; RAS: Renin-angiotensin system; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; STR: Short tandem repeat; PFA: Paraformaldehyde; H&E: Hematoxylin and eosin; PAS: Periodic acid Schiff; BSA: Bovine serum albumin; RT-PCR: Real-time PCR; VEGFR-2: Vascular endothelial growth factor receptor 2; AFP: Alpha-fetoprotein; PR: Partial response; TMA: Thrombotic microangiopathy; FSGS: Focal segmental glomerulopathy.

## **Declarations**

### **Acknowledgments**

We thank Prof. Ning Zhang (Vice president, Health Science Center, Peking University) for experimental support.

### **Author contributions**

T. Zhang, H.K. Li and H. Guo designed the experiments, acquired and analyzed the data, S. Zhang performed all experiments, S. Zhang, M.Q. Cao, Z.Y. Hou, X.Y. Gu, Y.Z. Chen, L. Chen, Y. Luo, L.W. Chen, D.M. Liu, H.Y. Zhou, K.Y. Zhu<sup>1</sup>, Z.W. Wang, X.H. Zhang, X.L. Zhu, Y.L. Cui analyzed the data. H.K. Li, S. Zhang, H. Guo and T. Zhang wrote the manuscript. All authors discussed and revised the manuscript.

### **Funding**

This study was supported by the National Natural Science Foundation of China (No. 81672884) and the National Science and Technology Major Project of China (No. 2017ZX10203207-004-005).

### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Ethics approval and consent to participate**

All human studies were approved by the Clinical Research Ethics Committee of Tianjin Medical University Cancer Institute. All animal experiments were carried out in accordance with a protocol approved by the ethics committee of the Institutional Animal Care of Tianjin Medical University Cancer Institute and Hospital.

### **Consent for publication**

Not applicable for this article.

### **Conflict of interest**

The authors have declared that no conflict of interest exists.

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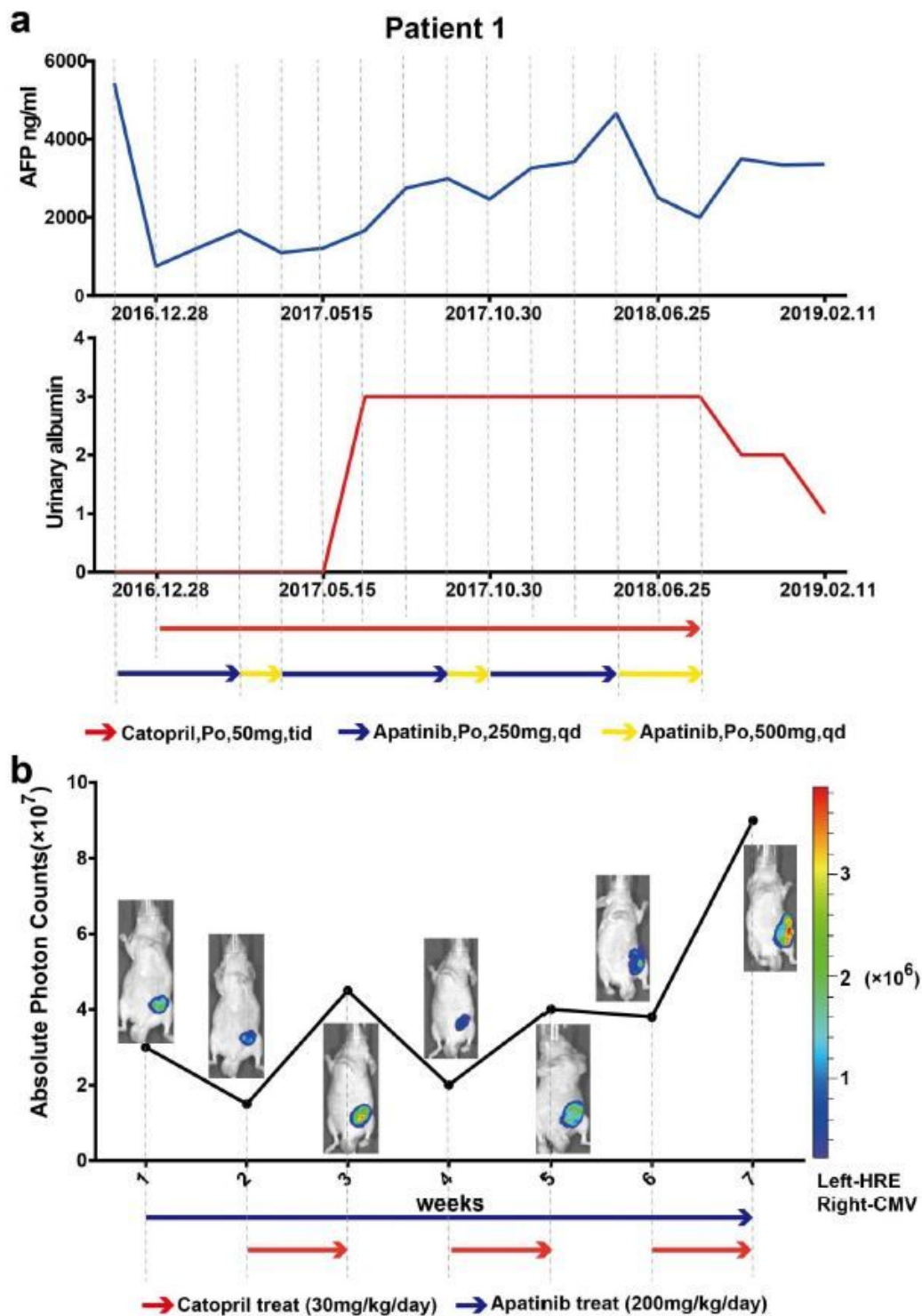
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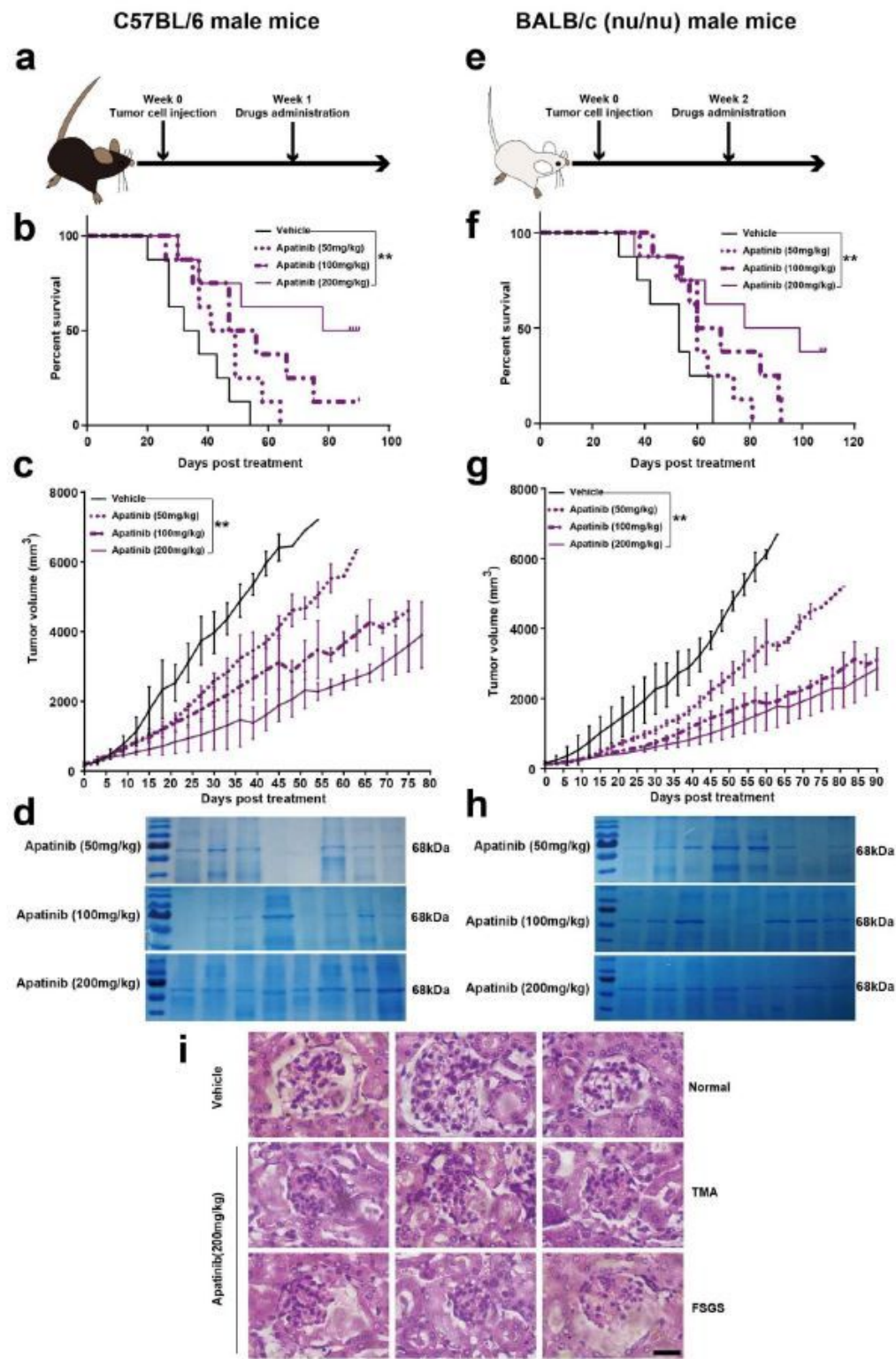
## Figures



**Figure 1**

In HCC patients and tumor-bearing mice, captopril compromises the anticancer effect of apatinib without reducing proteinuria. (a) Changes in AFP and urinary protein with the course of medication of Patient 1. (b) Quantification of representative bioluminescent images of mice with subcutaneous tumors formed by MHCC97H-Luc cells. Images were obtained after a week of continuous captopril administration or

withdrawal. Shown on the Y-axis are absolute photon counts for the MHCC97H-Luc tumor. The arrows in various colors represent the duration of the corresponding medication.



**Figure 2**

Determining the optimal dose of apatinib in two tumor-bearing mouse models and establishing a stable proteinuria model. a, b, c and d are the data of C57BL/6 mice, e, f, g and h are the data of BALB/c (nu/nu) mice. (a, e) Experimental design. (b, f) Survival curves of apatinib-treated subcutaneous tumor-bearing

mice (n = 8). (c, g) Measurement of tumor volume at different time points after apatinib administration. (n = 8, two-tailed t test). (d, h) Coomassie brilliant blue staining of 24-hour urine samples from each experimental group. (i) Representative images of hematoxylin-eosin (HE)-stained glomeruli. Scale bar, 50  $\mu$ m. \*p<0.01, \*\*p<0.001, ns represents no significant difference.

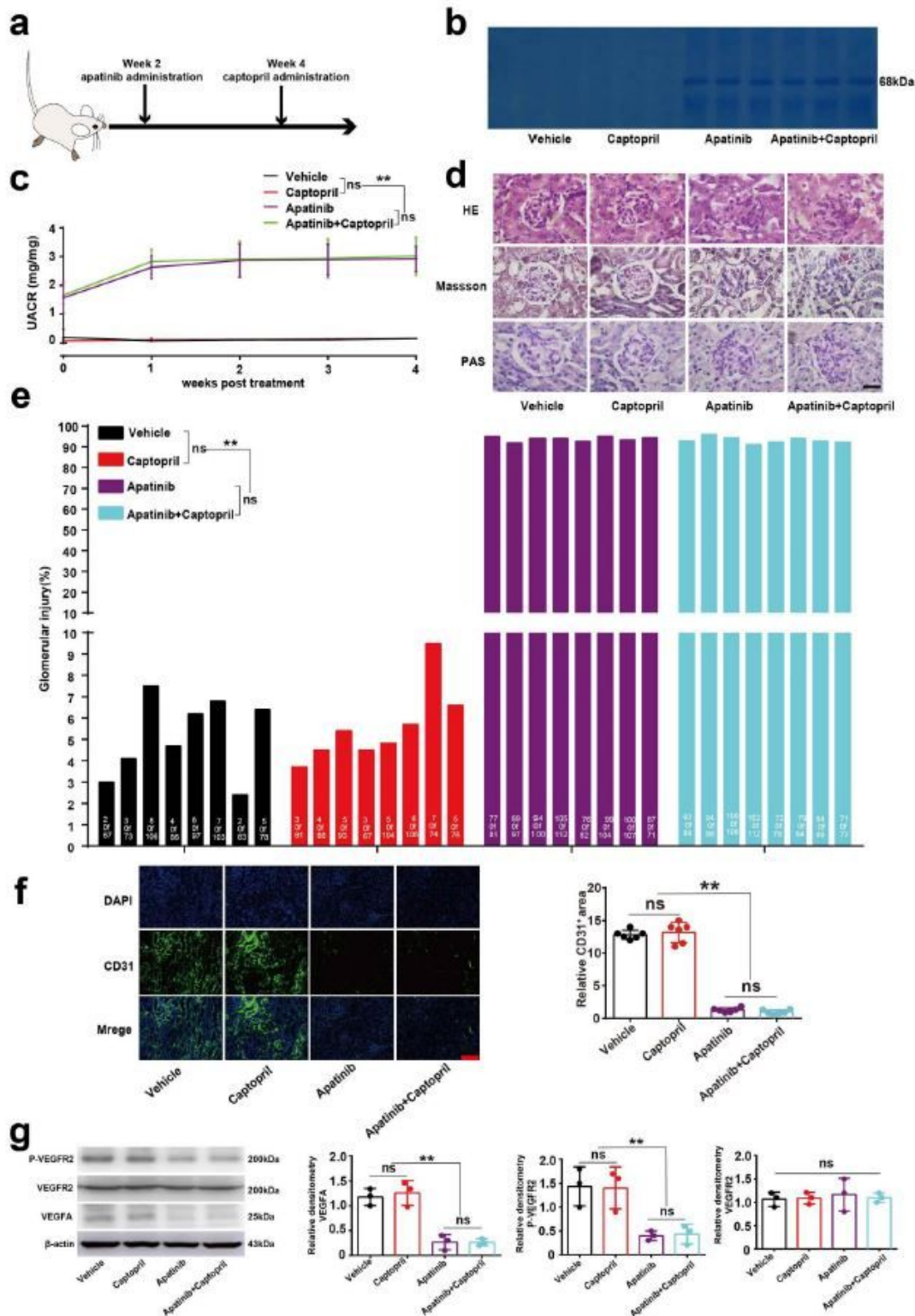
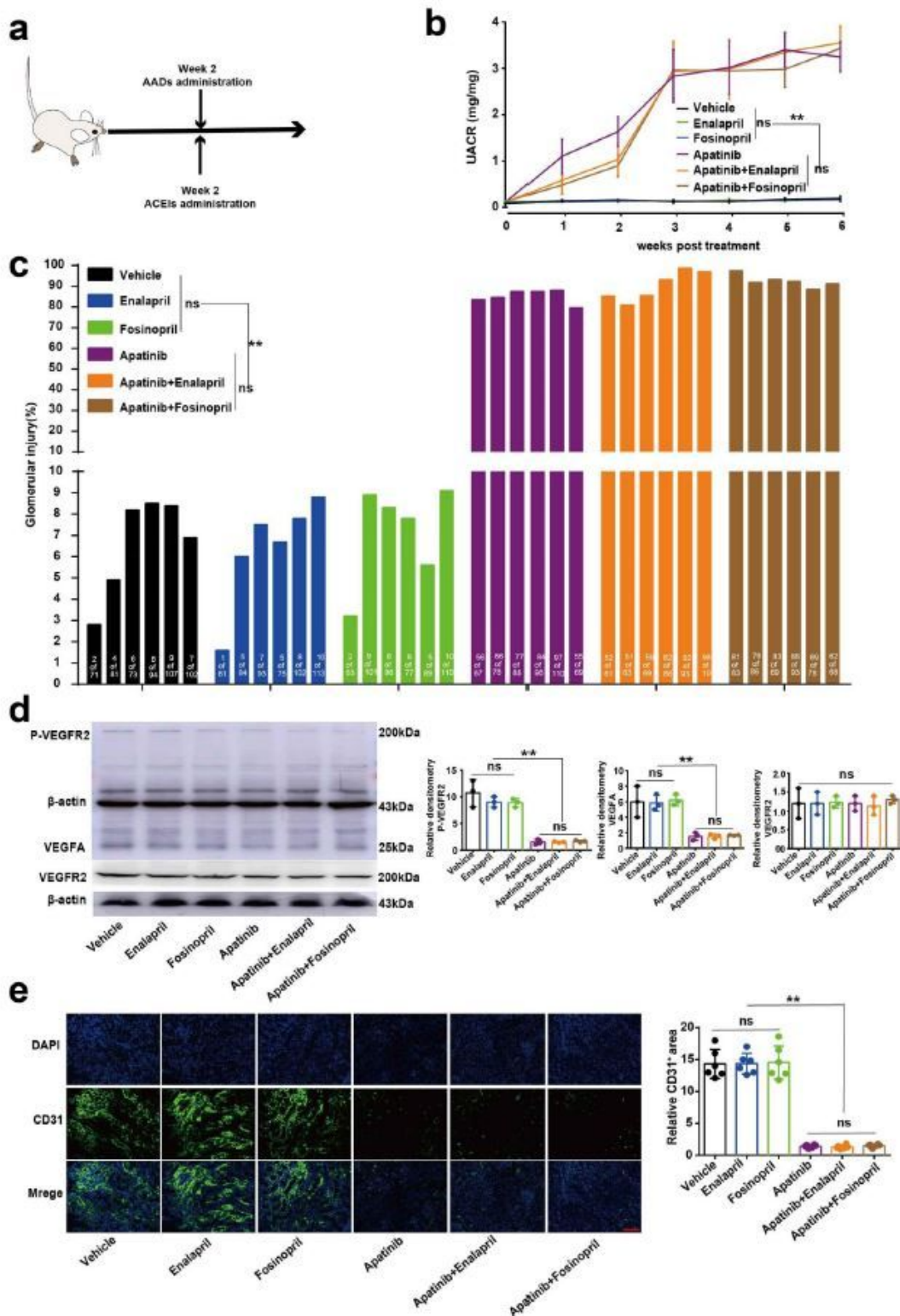


Figure 3

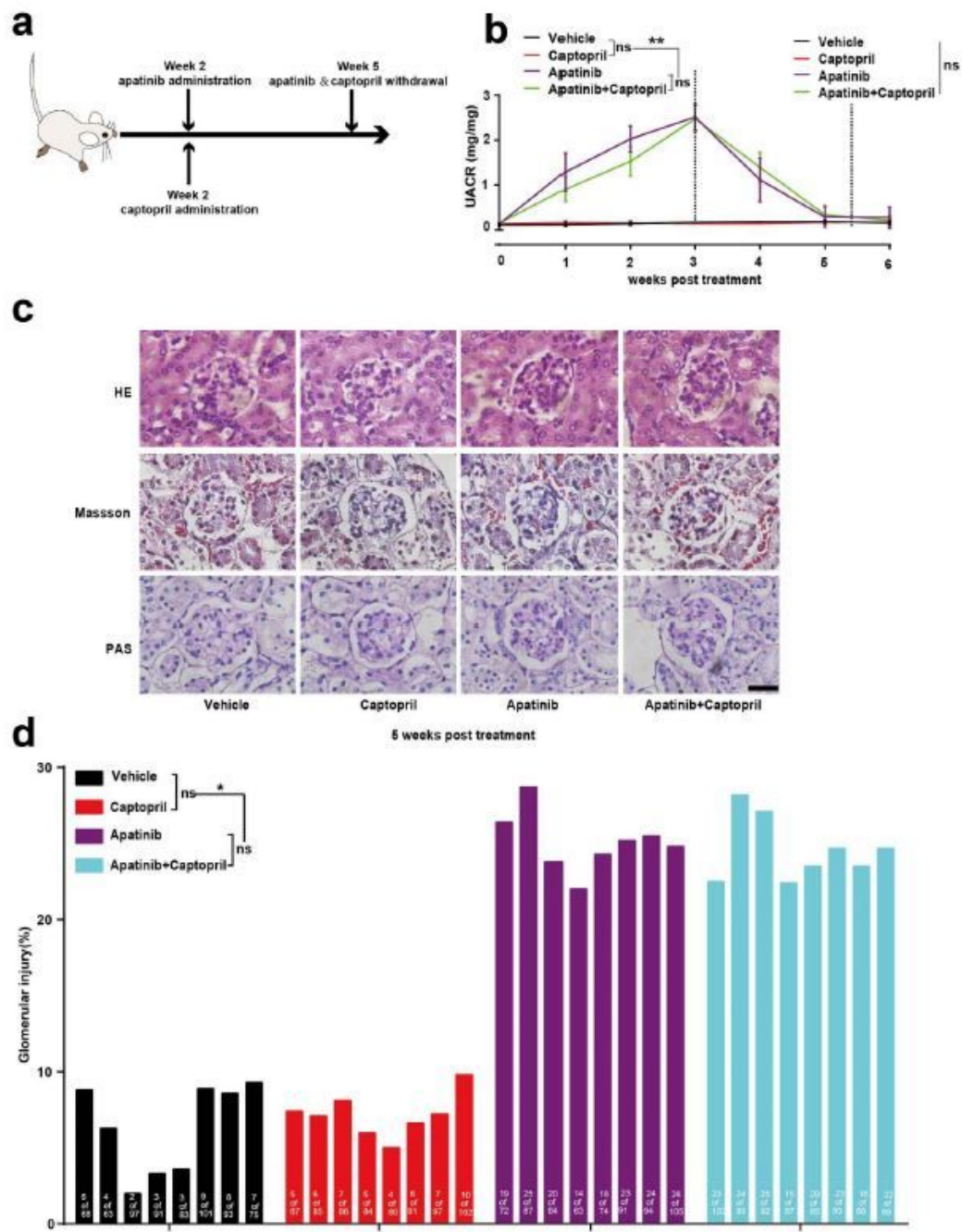
Captopril does not affect the VEGF signaling pathway, does not affect anti-angiogenesis, and does not reduce apatinib-related proteinuria or kidney damage. (a) Experimental design. (b) Coomassie gel showing albuminuria in each group of mice (10  $\mu$ l of urine in each lane). (c) Quantification of urinary albumin/creatinine (UACR). n=8 mice per group. (d) Representative images of hematoxylin-eosin (HE), periodic acid–Schiff (PAS) and Masson-stained kidneys. Masson staining and PAS staining are helpful for viewing thrombosis and glomerulosclerosis. Scale bar, 50  $\mu$ m. (e) Percentage of damaged glomerulus in each pathological section. (f) Kidney CD31+ microvessel signals of the mice. CD31+ microvessel signals were randomly quantified from 12 fields (n = 6 samples per group). (g) Western blotting analysis of VEGFA, VEGFR2 and P-VEGFR2 in mouse kidneys (n = 3 samples per group). \*p<0.01, \*\*p<0.001, ns represents no significant difference.



**Figure 4**

Enalapril and fosinopril do not affect the VEGF signaling pathway, do not affect anti-angiogenesis, and do not delay apatinib-related proteinuria or kidney damage. (a) Experimental design. (b) Quantification of urinary albumin/creatinine (UACR).  $n=8$  mice per group. (c) Percentage of damaged glomeruli in each pathological section. (d) Western blotting analysis of VEGFA, VEGFR2 and P-VEGFR2 in mouse kidneys ( $n$

= 3 samples per group). (e) Kidney CD31+ staining of the mice. CD31+ staining was randomly quantified from 12 fields (n = 6 samples per group). \*p<0.01, \*\*p<0.001, ns represents no significant difference.



**Figure 5**

Captopril does not affect the reduction in proteinuria or the recovery of kidney injury after apatinib withdrawal. (a) Experimental design. (b) Quantification of urinary albumin/creatinine (UACR). n=8 mice per group. (c) Representative images of HE, PAS and Masson-stained kidneys, Scale bar, 50  $\mu$ m. (d)

Percentage of damaged glomeruli in each pathological section. \*p<0.01, ns represents no significant difference. \*p<0.01, \*\*p<0.001, ns represents no significant difference.

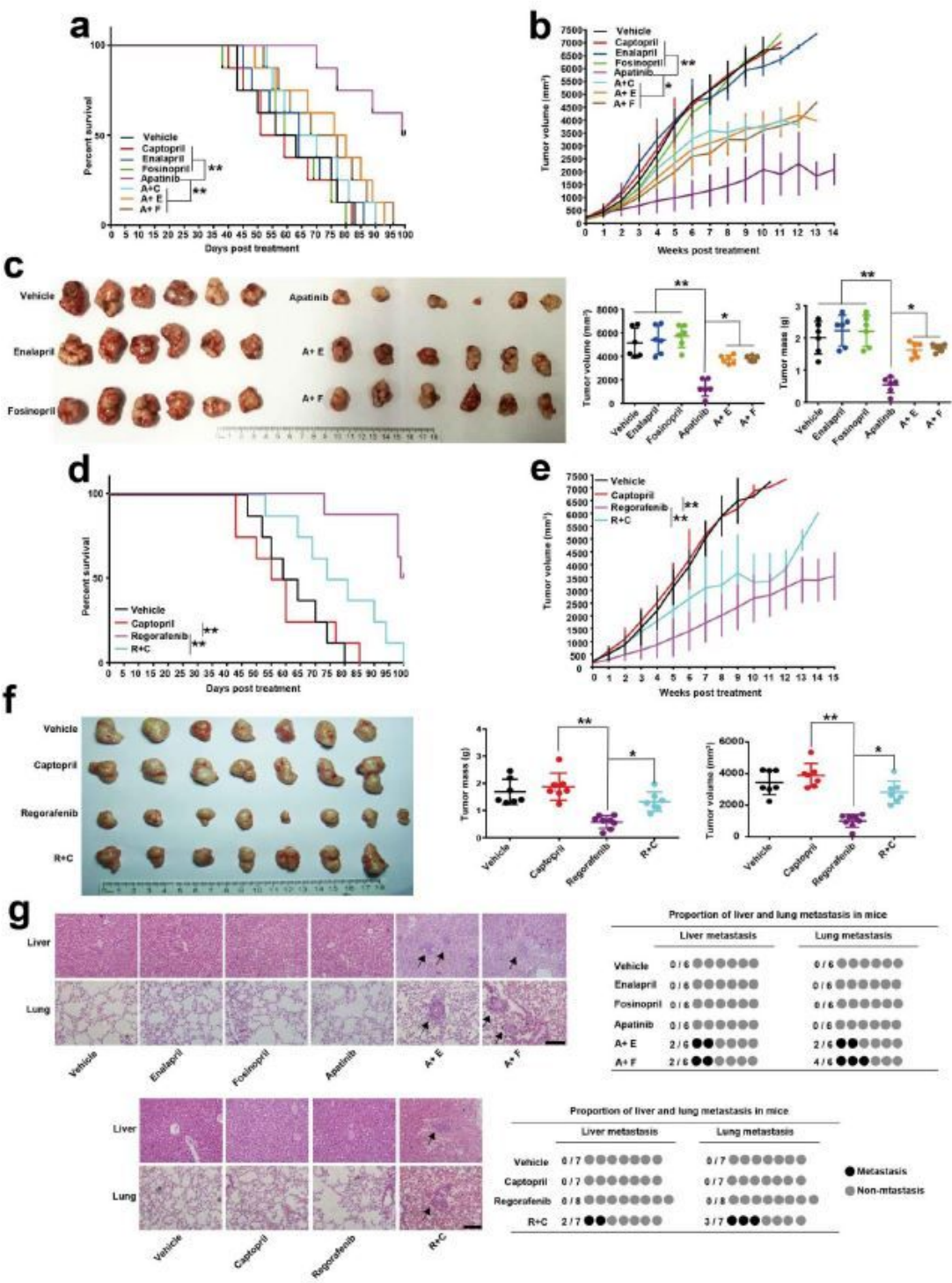


Figure 6

ACEIs abate the anticancer ability of AADs in tumor-bearing immunodeficient mice and promote liver and lung metastasis. Abbreviations: apatinib + captopril (A+C), apatinib + enalapril (A+E), apatinib + fosinopril (A+F) and regofenib + captopril (R+C). (a, d) Survival curves of subcutaneous tumor-bearing mice treated

with ACEIs alone, AADs alone, or a combination of both (n = 8). (b, e) Measurement of tumor volume at different time points after ACEIs alone, AADs alone, or a combination of both administration (n = 8). (c, f) Subcutaneous tumors and quantification of their mass and volume at certain time points (n =6, 7 or 8). (g) Representative images of HE-stained sections of the liver and lungs for subcutaneous tumor metastasis to the liver and lung and the number of mice with liver and lung metastasis. \*p<0.01, \*\*p<0.001, ns represents no significant difference.

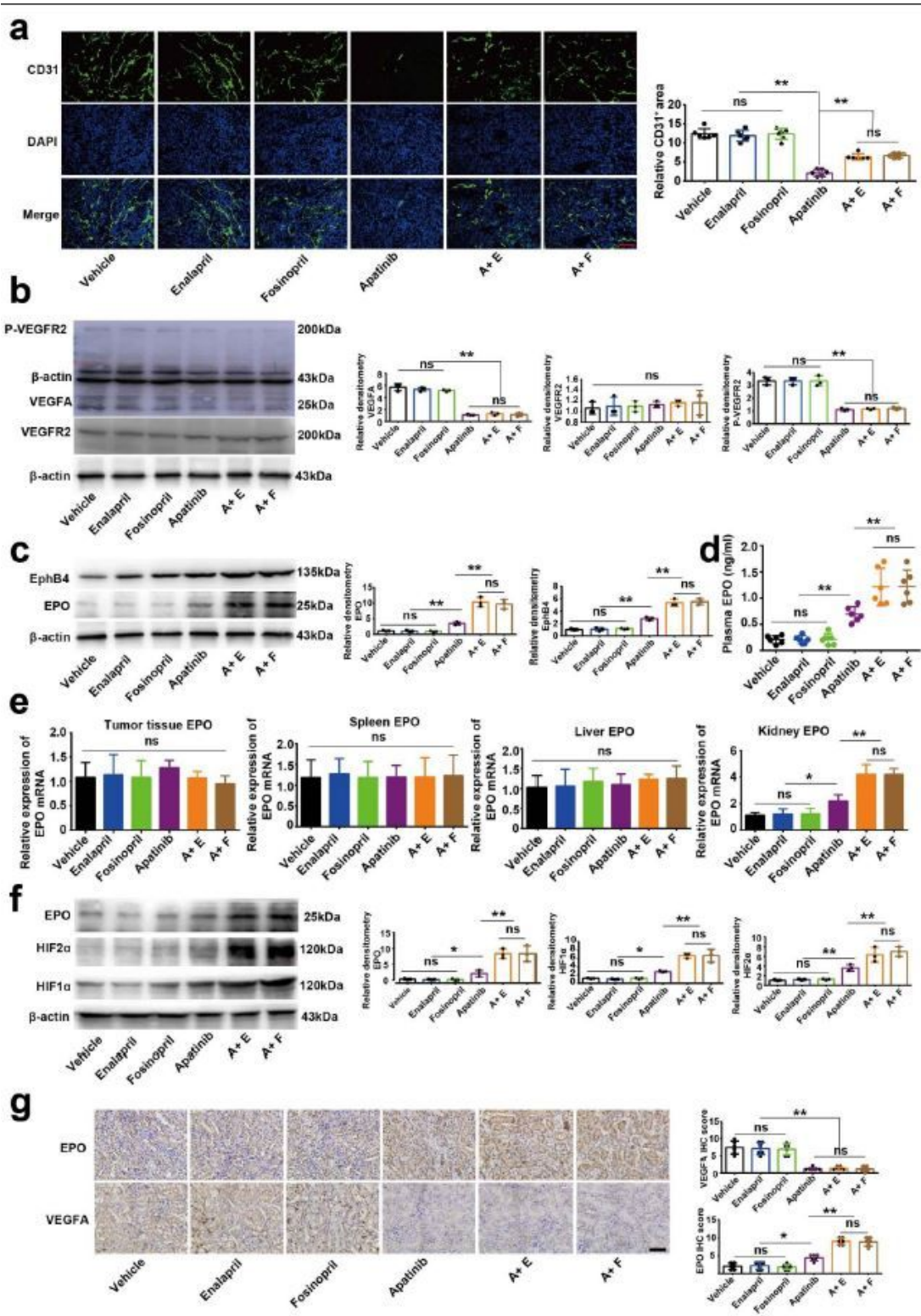
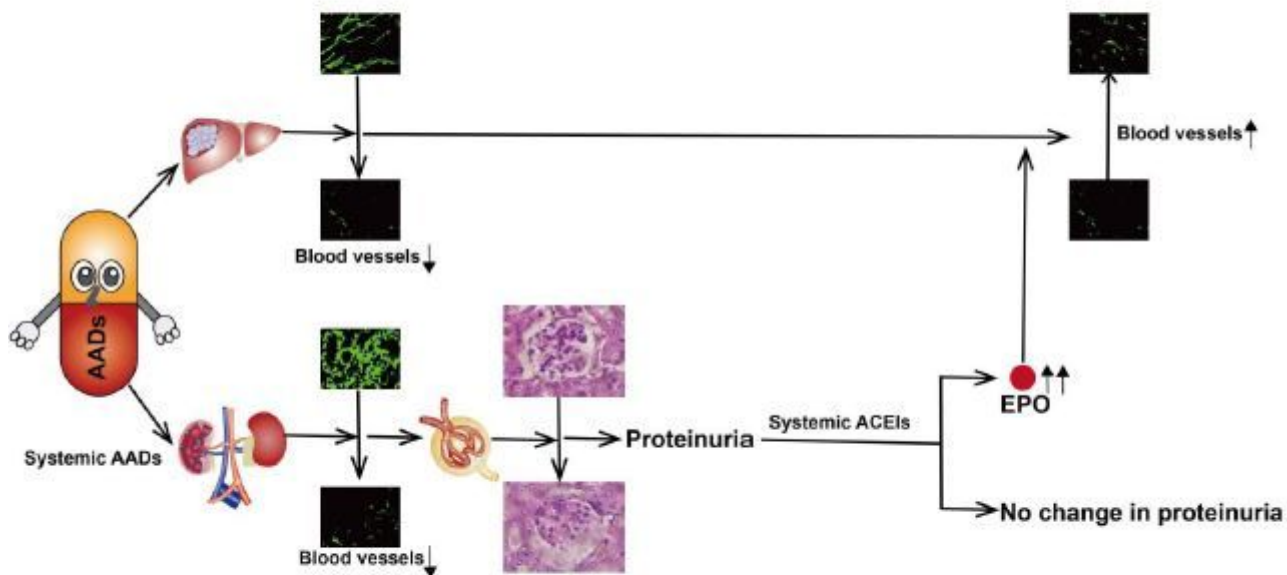


Figure 7

ACEIs promote the production of renal EPO and activate the tumor EPO signaling pathway under the treatment of AADs. (a) Tumor tissue CD31+ staining of mice treated with ACEIs alone, AADs alone, or a combination of both. CD31+ staining was randomly quantified from 12 fields (n = 6 samples per group). (b) Western blotting analysis of VEGFA, VEGFR2 and P-VEGFR2 in mouse tumor tissue (n = 3 samples per group). (c) Western blotting analysis of EPO and EphB4 in mouse tumor tissue (n = 3 samples per group). (d) ELISA measurements of EPO levels in circulating blood (n = 6 samples per group). (e) EPO mRNA levels of tumors in the spleen, liver, kidney and tumor of each group (n = 6 samples per group). (f) Western blotting analysis of EPO, HIF-1 $\alpha$  and HIF-2 $\alpha$  in mouse kidneys (n = 3 samples per group). (g) Representative immunohistochemical staining of EPO and VEGFA in the mouse kidney cortex and comparison of slice scores for each group (n = 6 samples per group). \*p<0.01, \*\*p<0.001, ns represents no significant difference.



**Figure 8**

Schematic diagram: ACEIs do not alleviate proteinuria or kidney damage caused by AADs but promote the resistance of AADs by increasing the production of kidney-derived EPO. Systemic administration of AADs such as apatinib enters the circulation to target both the tumor and kidney and cause a decrease in blood vessel density. AAD-induced vessel regression in the kidney leads to glomerular injury and proteinuria, and systemic administration of ACEIs cannot reverse the reduction in renal blood vessels and does not alleviate glomerular damage or proteinuria but instead induces large amounts of EPO

production in the renal cortical area. Kidney-derived EPO enters the circulation to target tumors, activates the EPO signaling pathway, resists anti-angiogenic effects and eventually contributes to AAD resistance. These findings support the notion that ACEIs are not recommended for the treatment of hypertension and proteinuria caused by AADs.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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