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Dose-dependent effects of the ACE inhibitor, ramipril, on kidney function and structure in the Alport COL4A3$^{-/-}$ mouse

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Abstract

Introduction: ACE inhibitor treatment of Alport syndrome, undertaken when individuals are still presymptomatic/oligosymptomatic delays dialysis but substantial residual risk remains so that additional treatments are desperately needed. In contrast to humans, standard-dose ACE inhibition with ramipril 10 mg/kg/day in Alport mice almost completely prevents GFR loss, precluding the ability to test new add-on therapies. Accordingly, we sought to find a dose that mirrored the ~50% improvement in GFR seen in humans.

Material and methods: Escalating doses of ramipril at 0.1, 1, 3 and 10 mg/kg/day, were administered to COL4A3$^{-/-}$ mice.

Results: After four weeks, ramipril induced a logarithmic, dose-dependent decline in cystatin C, the primary GFR-based outcome, with efficacy apparent at 0.1 mg/kg/day and a 50% improvement at 1–3 mg/kg/day. Proteinuria, the study's secondary outcome, showed a similar dose-response. In contrast, structural improvements such as podocyte density, glomerulosclerosis and tubulointerstitial fibrosis were not evident below 3 mg/kg/day.

Conclusion: By exploring the dose-response relationship of RAS blockade in an Alport model, the present study shows that ramipril 1 mg/kg/day may be sufficient for examining additive effects of other agents on kidney function while 3 mg/kg/day is required in order to assess the effects of add-on therapy on kidney structure as well.

Keywords

Alport syndrome, glomerular filtration rate, proteinuria, renin-angiotensin system, podocyte, glomerulosclerosis, tubulointerstitial fibrosis

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Introduction

Alport syndrome is an inherited disease of basement membrane collagen that affects the kidney, eye and cochlea, and accounts for around 1.5–3% of children and young adults with end-stage kidney disease in Europe and the U.S.[1] Unlike most other forms of progressive kidney disease that present with advanced disease that is often sporadic and multifactorial in aetiology, children with a family history of Alport syndrome can be diagnosed in the first decade of life when the disease in its early oligosymptomatic stage (microscopic haematuria ± microalbuminuria) or even before with the help of genetic testing.[2,3] As such, early, presymptomatic therapeutic intervention is not only desirable but has been shown to be effective.[4]

In the EARLY PRO-TECT trial, Gross et al. showed that the angiotensin converting enzyme (ACE) inhibitor, ramipril, when administered to children with early, oligosymptomatic Alport syndrome reduced the rate of GFR decline by approximately half along with a substantial diminution in the progression of albuminuria.[4] The resultant attenuation in kidney function loss delayed end-stage kidney disease and the requirement of dialysis or transplantation to preserve life by approximately 13 years. Accordingly, ACE inhibition is now standard of care in Alport syndrome and

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while such treatment means that severely affected individuals start dialysis at a median age of 40 rather than in their 20s, additional treatments are desperately needed.

Preclinical testing in animal models is a key component in the development of new therapies. To date, the maximal dose of an ACE inhibitor used in human clinical trials has been ramipril 10 mg/day,[4] while in the commonly used Alport COL4A3$^{-/-}$ mouse model the maximal dose of this drug was 10 mg/kg/day with which GFR loss was almost completely prevented.[5] This near total abrogation of disease in the mouse, precludes the ability to test the efficacy of new add-on therapies in a prophylactic setting.

In order to establish a preclinical model for testing the efficacy of early intervention with the potential for subsequent combination therapy studies, the present study sought to find a dose of ramipril that mirrored the ~50% improvement in humans with oligo-/asymptomatic Alport syndrome. To bolster its relevance to the human scenario, we selected, *a priori*, primary, secondary and tertiary outcomes that might be used in a pivotal clinical trial. Accordingly, the primary outcome centred on GFR, the secondary outcome focussed on proteinuria and the tertiary, more exploratory outcome sought to examine changes in the principal histological manifestations of Alport syndrome of glomerulosclerosis, podocyte loss and tubulointerstitial fibrosis.

Methods

Animals

With its ability to provide a robust phenocopy of Alport syndrome in humans, a highly reproducible timeline of progression and responsiveness to nephroprotective therapies akin to that seen in patients with the disease,[6] the COL4A3 mouse was chosen for the current study. Moreover, with previous published reports showing similar pathology and disease progression in female and male COL4A3 mice both sexes were used.[7] Heterozygous COL4A3 knockout mice (129-*Col4a3^{tm1Dec}*/J, The Jackson Laboratory, Farmington, CT) were genotyped by ear biopsy, DNA extraction and PCR prior to crossbreeding as previously reported.[8] Genotyping was then repeated just prior to randomization at four weeks of age. After genetic confirmation, homozygous COL4A3$^{-/-}$ mice were randomized to receive vehicle (reverse osmosis [RO] water), or ramipril at a dose of 0.1, 1, 3 or 10 mg/kg in RO water for four weeks in drinking water, calculated using the formula:

$$[\text{ramipril}] = \text{target dose}(\text{mg / kg / day}) \times \text{body weight (kg) / estimated water intake} \; (0.25\ \text{ml / g body weight / day}),$$

the choice of ACE inhibitor reflecting ramipril's extensive prior use in both mouse and human studies of Alport syndrome.[4,5]

Treatment was started at 4 weeks of age corresponding to the onset of the structural and functional pathological manifestations of disease in COL4A3-/- mice[8] and continued for 4 weeks, after which a precipitous increase in mortality becomes problematic.[9]

Homozygous wild type mice from the same breeding colony drinking RO water served as controls. Mice (3–5/cage) were housed in a temperature-controlled room (22°C) with a 12-h:12-h light: dark cycle with free access to food and water. All animal studies were approved by the St Michael's Hospital Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publ. no. 85-23, revised 1996).

Just prior to termination a spot urine specimen was obtained. Terminal blood collection was performed via cardiac puncture after animals were anesthetized with 2–5% isoflurane, and a 4–5 cm incision was made in the anterior abdominal wall, kidneys were excised, capsules removed, weighed, and each kidney was bisected sagittally through the hilum to obtain two equal halves. One half was snap-frozen in liquid N_2 prior to storage at −80°C; the other half was fixed in 10% formalin for histology.[10]

Biochemistry

Plasma cystatin C, a marker of glomerular filtration rate, was assayed by ELISA (Mouse Cystatin C (CST3) ELISA Kit #EMCST3, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and expressed as ng/mL. Urinary albumin and creatinine concentrations were measured using colorimetric assay kits according to the manufacturer's instructions with urinary albumin quantified using the QuantiChrom™ BCG Albumin Assay Kit (BioAssay Systems, Hayward, CA; #DIAG-250), and urinary creatinine measured using the QuantiChrom™ Creatinine Assay Kit (BioAssay Systems; Cat. #DICT-500). The urinary albumin-to-creatinine ratio (uACR) was then calculated and expressed as micrograms of albumin per millimole of creatinine (μg/mM).

Immunohistochemistry

Immunohistochemistry was conducted using an automated system. In brief, deparaffinization, rehydration and target retrieval were done in a single step using an integrated immunohistochemistry pre-treatment module (Dako PT Link, Agilent, Santa Clara, CA 95051). Sections were then stained using an automated open system stainer (Dako Autostainer Link 48) according to the manufacturer's protocols prior to mounting and attaching coverslips. Immunolabelling was conducted for type 1 collagen using polyclonal goat anti-type 1 collagen antibody (#1310-01, Southern Biotech, Birmingham, AL) with secondary polyclonal rabbit anti-goat IgG (#305-035-003, Jackson ImmunoResearch (West Grove, PA) and glomerular epithelial cells (podocytes) using mouse monoclonal anti-human p57 Kip2 (KP39) antibody (#56341, Santa Cruz, Dallas, TX) with goat anti-mouse as secondary (#115-035-003, Jackson ImmunoResearch). Nuclei were labelled by counterstaining with hematoxylin (Dako, #CS700). Immunoperoxidase staining was performed as reported previously[11] using horseradish peroxidase (HRP)-conjugated secondary antibodies with peroxidase activity indicated by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Dako) as substrate.

Immunostaining quantification

Stained sections were scanned (Zeiss, Axio Scan.Z1, White Plains, NY), and analyzed using a quantitative digital pathology platform (HALO, Indica Labs, Albuquerque, NM).

For collagen 1 the extent of immunolabelling was expressed as the proportional area demonstrating positive staining. The kidney cortex and medulla were analyzed separately. Podocyte counting was performed as previously described.[12,13] In brief, 80 glomeruli were annotated using the HALO image analysis platform with the total glomerular area determined accordingly. p57-positive nuclei were counted manually using a hemocytometer with the observer masked to sample identity. Data were expressed as the number of p57-positive cells per 1000 μm^2 of glomerular area.

Glomerulosclerosis index

Tissue sections were stained with Periodic acid-Schiff (PAS) with the extent of glomerulosclerosis quantified, as previously reported.[14] In brief, glomerulosclerosis was assessed using a semi-quantitative technique in a masked fashion counting 80 randomly selected glomeruli for each animal. The extent of sclerosis in each glomerulus was graded on a scale of 0–4: Grade 0, normal; Grade 1, sclerotic area up to 25% (minimal); Grade 2, sclerotic area 25–50% (moderate); Grade 3, sclerotic area 50–75% (moderate to severe) and Grade 4, sclerotic area 75–100% (severe). A glomerulosclerotic index (GSI) was then calculated using the formula:

$$GSI = \sum_{(i)=0}^{4} Fi\,(i)$$

Where Fi is the percentage of glomeruli in the animal with a given score (i).

Statistics

Data are expressed as means ± SEM with individual data points unless otherwise specified. Between group differences were analyzed by one way ANOVA with Fisher's Protected Least Significant Difference test *post hoc*. All statistics were performed using GraphPad Prism 10.4.2 for Mac OS X (GraphPad Software Inc., San Diego, CA). A p value of <0.05 was regarded as statistically significant.

Results

Kidney function

Plasma cystatin C was markedly elevated in COL4A3$^{-/-}$ mice when compared with wild type controls (Figure 1). The administration of ramipril led to a dose-dependent reduction in cystatin C that was evident at the lowest dose of 0.1 mg/kg/day. At 10 mg/kg/day, ramipril effectively normalized kidney function in COL4A3$^{-/-}$ mice with no discernible statistical significance in cystatin C between KO mice receiving this dose and WT mice ($p = 0.3333$).

Like its effects on cystatin C, escalating doses of ramipril induced a similar, albeit steeper, logarithmic decline in the urinary albumin: creatinine ratio (uACR, Figure 2). Once again, there was no discernible statistical significance in the uACR between KO mice receiving ramipril 10 mg/kg/day and WT mice ($p = 0.8715$).

Glomerulosclerosis

Untreated COL4A3$^{-/-}$ mice developed marked glomerulosclerosis (Figure 3). In contrast to the functional changes induced by low dose ramipril, no significant change in the extent of glomerulosclerosis was observed at ramipril doses <3 mg/kg/day. However, similar to the dose-response relationship for cystatin C and uACR, 10 mg/kg/day ramipril profoundly reduced the extent of glomerulosclerosis rendering it indistinguishable from normal, control WT mice ($p = 0.7227$).

Interstitial collagen deposition

When compared with wild-type animals untreated COL4A3$^{-/-}$ mice developed marked collagen deposition in both the cortex and medulla (Figure 4). At 3 mg/kg/day ramipril reduced the abundance of immunostainable type 1 collagen in both the cortex and medulla. Unlike the extent of glomerulosclerosis, however, no incremental reduction was seen when the ramipril dose was increased up to 10 mg/kg/day. Lower doses were ineffective at reducing collagen 1 deposition. While there was no statistical difference in the extent of collagen deposition between control WT mice and those receiving 10 mg/kg/day ramipril, a trend was apparent ($p = 0.0963$) in the kidney cortex but not in the medulla ($p = 0.5093$).

Podocyte number

Podocyte numbers were dramatically reduced in untreated KO mice in comparison with WT mice (Figure 5). The

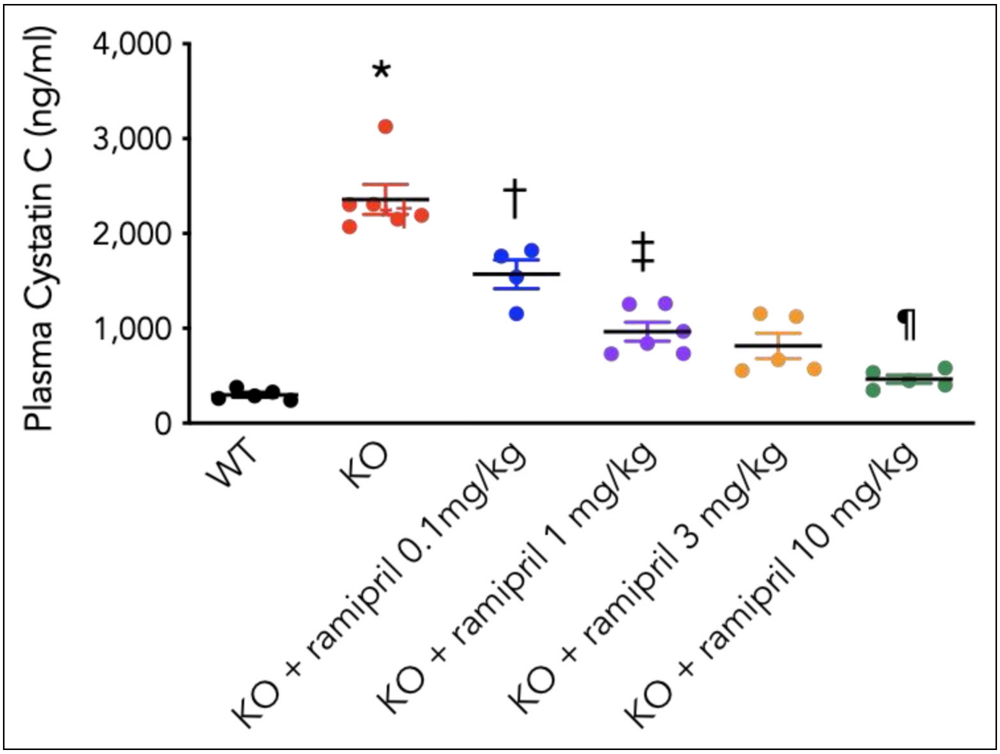

Figure 1. Graph showing the effects of varying ramipril doses on cystatin C demonstrating a reduction in plasma concentration with escalating doses of the drug. * $p < 0.0001$ versus wild type mice (WT); † $p < 0.0001$ versus untreated knockout KO mice; ‡ $p = 0.0015$ COL4A3$^{-/-}$ (KO) mice receiving ramipril 1 versus 0.1 mg/kg/day; ¶ $p = 0.0448$ KO receiving ramipril 10 versus 0.1 mg/kg/day. Animal numbers were as follows: WT (n = 5); KO (n = 6); KO + ramipril 0.1 mg/kg (n = 4); KO + ramipril 1 mg/kg (n = 6); KO + ramipril 3 mg/kg (n = 5); KO + ramipril 10 mg/kg (n = 5).

podocyte count remained similarly low at 0.1 and 1.0 ramipril mg/kg/day improving significantly when the dose was increased up to 3 mg/kg/day though with no further incremental improvement at 10 ramipril mg/kg/day.

Discussion

Animal models of disease serve a pivotal role in exploring disease pathophysiology and preclinical testing for the safety and efficacy of novel interventions before their use in humans. Unfortunately, the translatability of findings in animal models to the human context is fraught, not least because of the lack of disease commonality between the human disease and the rodent counterpart used to study it.[15] In contrast to the vast majority of instances, however, the COL4A3$^{-/-}$ mouse model of Alport syndrome closely recapitulates the biochemical and histological manifestations of disease seen in males with the X-linked form of the disease and homozygotes with the autosomal recessive variety that together account for 95% of cases.[1] While the administration of a preclinical intervention is usually best tested in the therapeutic setting so as to match that of patient setting where disease is already well- established, the scenario in Alport syndrome is fundamentally different. Here, given its heredity origin, cases can be identified at a very young age in the pre- or oligosymptomatic stage with the ability to conduct animal studies that reflect these circumstances, as in the present report.

A key strength of the current study is its relevance to the translational setting of early, asymptomatic or oligosymptomatic Alport syndrome with its the use of a clinically relevant marker of kidney function as its pre-designated, primary outcome. By establishing a dose of ramipril 1 mg/kg/day that mirrors the effects of standard of care in the clinical setting with an approximate halving of the GFR loss, the study provides guidance for testing the effectiveness of additional therapies in the preclinical context. Indeed, several potential add-ons to ACE inhibition such as endothelin antagonists, mineralocorticoid blockers and SGLT2 inhibitors and are already in use for other forms of chronic kidney disease[2] and could be tested preclinically for their potential use in Alport syndrome.

Glomerular filtration rate, the primary outcome in the current study, as it is in phase 3 clinical trials, provides a surrogate endpoint for the development of end-stage kidney disease in the clinic. Of the three commonly used markers of GFR: serum creatinine, BUN and cystatin C, the latter, though slightly more expensive and so less widely used is

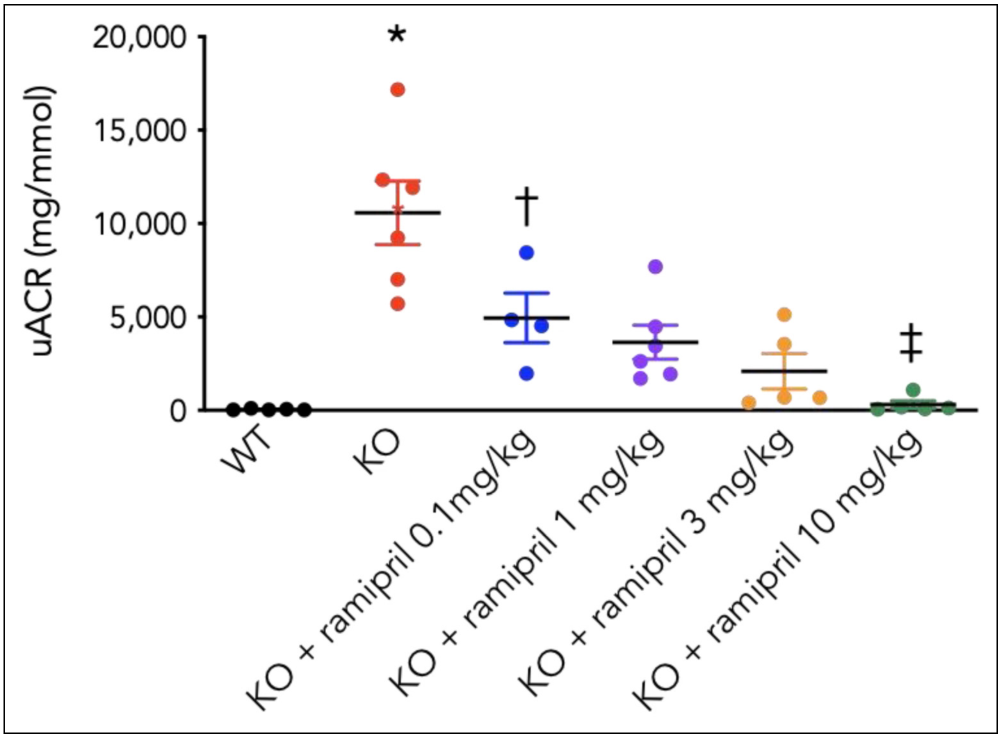

Figure 2. Effects of varying ramipril doses on the urinary albumin: creatinine ratio (uACR) showing a reduction in ratio with escalating doses of the drug. * $p < 0.0001$ versus wild type mice (WT); † $p = 0.0016$ versus untreated knockout KO mice; ‡ $p = 0.0095$ KO receiving ramipril 10 versus 1 mg/kg/day. Animal numbers were as follows: WT (n = 5); KO (n = 6); KO + ramipril 0.1 mg/kg (n = 4); KO + ramipril 1 mg/kg (n = 6); KO + ramipril 3 mg/kg (n = 5); KO + ramipril 10 mg/kg (n = 5)

commonly regarded as the most reliable in both humans[16] and laboratory mice.[17] Creatinine for instance, is influenced by muscle mass, age, sex, diet and race while BUN is modified by protein intake, hydration and liver function. Cystatin C, on the other hand, is made by all nucleated cells at a constant rate, is freely filtered by the glomerulus, is neither absorbed nor secreted by kidney tubules and is unaffected by muscle mass, diet or sex. Accordingly, and in the context of the low body weights, reduced muscle mass and food intake, Cystatin C was selected as the preferred marker of GFR in the present study.

Reduction in proteinuria, the secondary outcome in this study, was assessed by changes in urinary ACR that like cystatin C showed a logarithmic dose-response relationship identifiable at 0.1 mg/kg/day of ramipril. This low dose is, notably, 1/100th of the dose used in previous studies of this drug in Alport COL4A3$^{-/-}$ mice in both the prophylactic and therapeutic settings.[5,6] And while not the primary outcome of large, registration trials in kidney disease, reduction in proteinuria can provide an early marker of therapeutic efficacy.

While the renin-angiotensin system (RAS) is traditionally viewed as a classical endocrine pathway, the kidney also has a localized intrarenal counterpart that functions quasi-independently from it[18] with concentrations of angiotensin II that are 10- to 1000-fold higher in the glomerular filtrate, tubular fluid and interstitium than in plasma.[19,20] Indeed, given the major improvement in cystatin C and uACR at only 0.1 mg/kg/day ramipril the findings of the present study suggest that the intra-renal RAS rather than its endocrine counterpart may be the key mediator of podocyte injury, mononuclear cell infiltration and fibrogenesis.[18] Of note, such findings are similar to a study in the streptozotocin-induced diabetic (mREN-2)27 rat in which perindopril when micro-dosed at 0.02, 0.2 as well as 2 mg/kg/day exerted beneficial effects analogous to the current study in Alport mice.[21]

In Alport syndrome, mutations in the gene coding for α3, α4 and α5 chains of type IV collagen result in changes to the collagen IV$^{\alpha345}$ heterotrimer, major component of the glomerular basement membrane.[22] These changes, in turn, lead to dysfunction of the glomerular basement membrane with disrupted barrier function and podocyte detachment that in turn lead to glomerulosclerosis.[23]

While tubulointerstitial disease is also a well-recognized feature of Alport syndrome it is thought to develop indirectly since the collagen IV$^{\alpha345}$ heterotrimer is confined to the glomerular basement membrane.[24] Studies showing that albumin deficient mice with Alport syndrome develop less interstitial fibrosis and tubular atrophy suggest that tubulointerstitial pathology in Alport syndrome develops as a consequence of the progressive proteinuria that

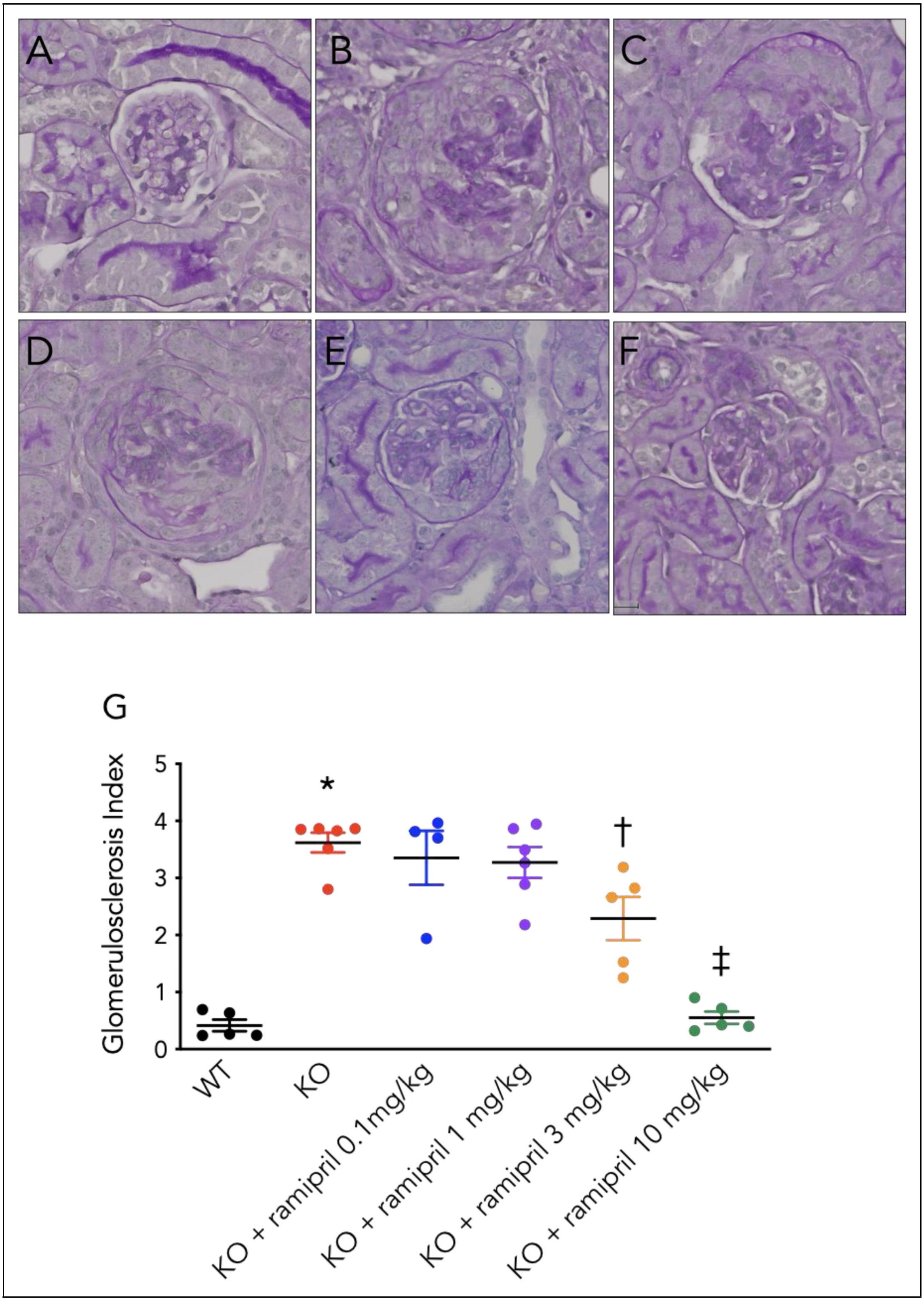

Figure 3. Photomicrographs of representative images from PAS-stained sections of control mice (A) and KO mice treated with either vehicle (B), 0.1 (C), 1 (D), 3 (E), or 10 mg/kg/day ramipril (F). Magnification 200×. Graph showing the extent of glomerulosclerosis (H). At ramipril 3 mg/kg/day the magnitude of glomerulosclerosis was reduced in KO mice with a further reduction at 10 mg/kg/day. Doses lower than 3 mg/kg/day were ineffective at reducing glomerulosclerosis. * $p < 0.0001$ versus wild type mice (WT); † $p = 0.0012$ versus untreated knockout mice; ‡ $p = 0.0001$ KO receiving ramipril 10 versus 3 mg/kg/day. Animal numbers were as follows: WT (n = 5); KO (n = 6); KO + ramipril 0.1 mg/kg (n = 4); KO + ramipril 1 mg/kg (n = 6); KO + ramipril 3 mg/kg (n = 5); KO + ramipril 10 mg/kg (n = 5)

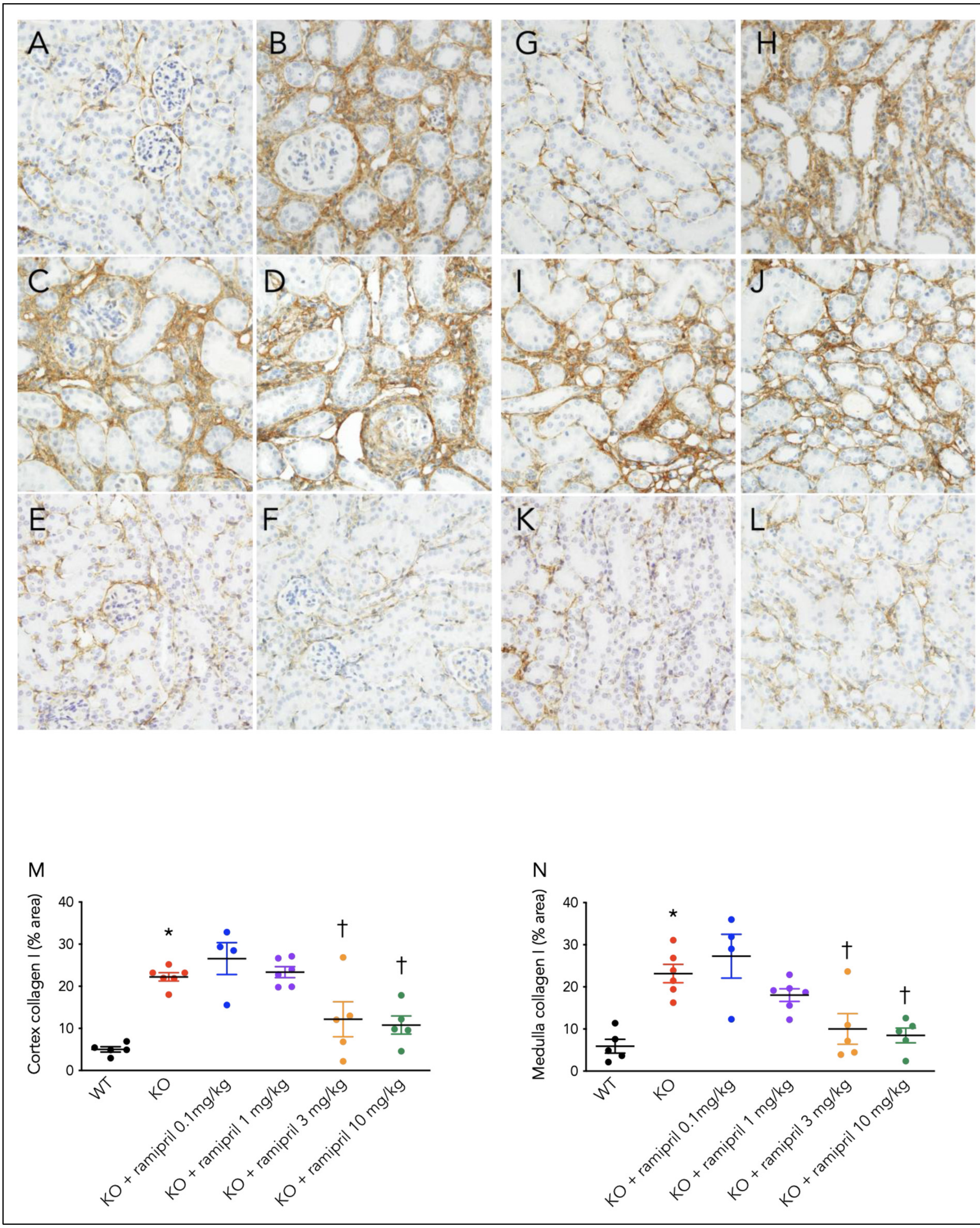

Figure 4. Photomicrographs of representative images from collagen 1-immunostained sections from the cortex (A-F) and medulla (G-L) of control mice (A, G) and KO mice treated with either vehicle (B, H), 0.1 (C, I), 1 (D, J), 3 (E, K), or 10 mg/kg/day ramipril (F, L). Magnification 40×. Graph showing the extent of collagen 1 deposition in the cortex (M) and medulla (N). Untreated KO mice displayed an approximate 5-fold increase in collagen 1 deposition when compared with WT mice. At 3 mg/kg/day ramipril reduced the abundance of immunostainable type 1 collagen in both the cortex and medulla. No incremental reduction was seen when the ramipril dose was increased up to 10 mg/kg/day. Lower doses were ineffective at reducing collagen 1 deposition. * $p < 0.0001$ versus wild type mice (WT); † $p < 0.01$ untreated knockout mice. Animal numbers were as follows: WT (n = 5); KO (n = 6); KO + ramipril 0.1 mg/kg (n = 4); KO + ramipril 1 mg/kg (n = 6); KO + ramipril 3 mg/kg (n = 5); KO + ramipril 10 mg/kg (n = 5)

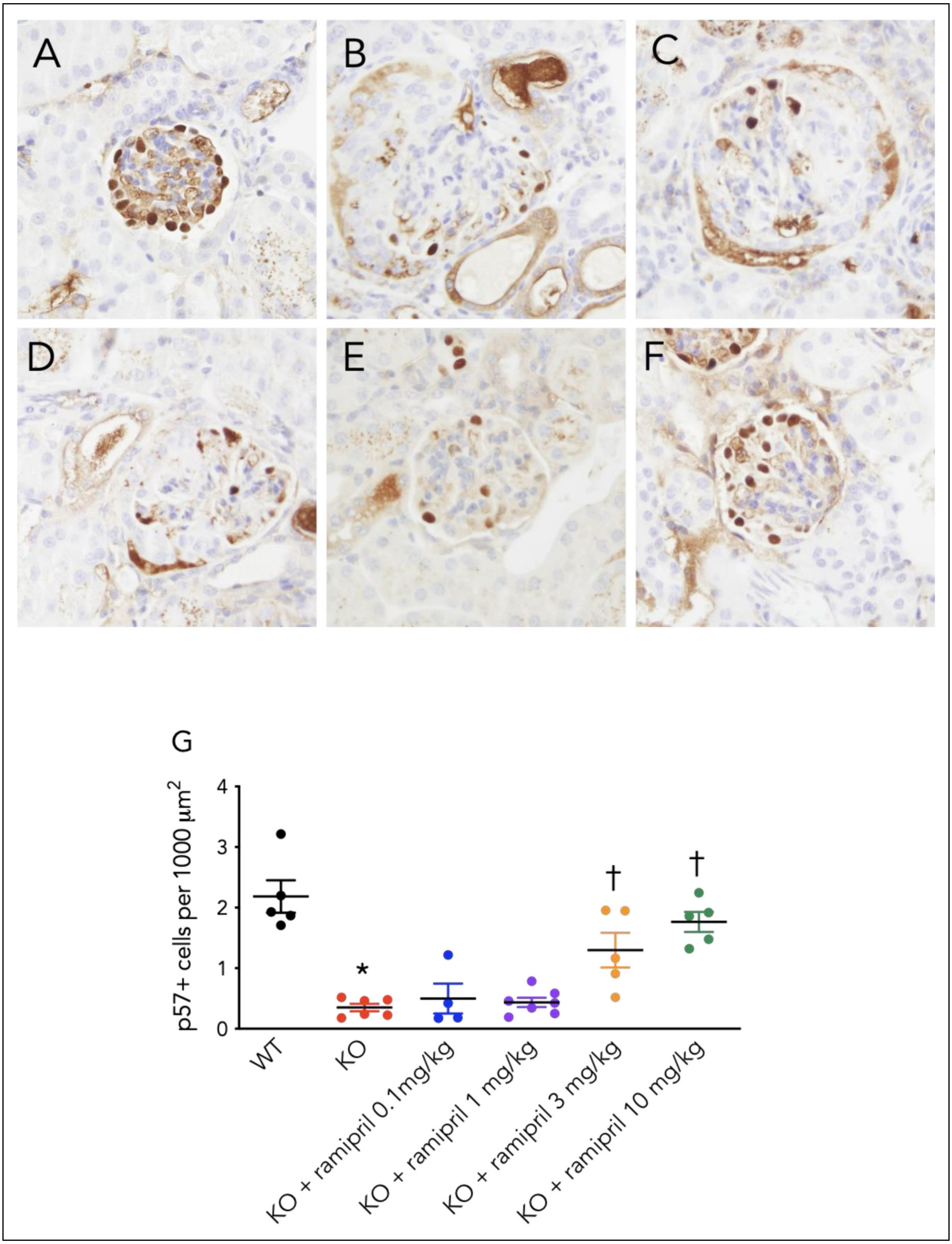

Figure 5. Photomicrographs of representative images from p57-immunostained sections from the cortex of control mice (A) and KO mice treated with either vehicle (B), 0.1 (C), 1 (D), 3 (E), or 10 mg/kg/day ramipril (F). Magnification 200×. Graph showing effects of varying ramipril doses on p57 immunolabelled podocytes (G). Untreated KO mice displayed marked reduction in the number of podocyte when compared with WT mice. At 3 mg/kg/day ramipril increased the number of p57 immunolabelled podocytes. No further increase was seen when the ramipril dose was increased up to 10 mg/kg/day. Lower doses were ineffective. * $p < 0.0001$ versus wild type mice (WT); † $p \leq 0.001$ untreated knockout mice. Animal numbers were as follows: WT ($n = 5$); KO ($n = 6$); KO + ramipril 0.1 mg/kg ($n = 4$); KO + ramipril 1 mg/kg ($n = 6$); KO + ramipril 3 mg/kg ($n = 5$); KO + ramipril 10 mg/kg ($n = 5$).

characterizes the disease rather than a primary defect in the tubular basement membrane.[25] In contrast to the improvement in cystatin C and ACR that began at the lowest dose of ramipril 0.1 mg/kg/day used, an accompanying reduction in structural injury was not evident below the 3 mg/kg/day. The mechanisms underlying the difference in ramipril dose required for improving kidney structure compared with its function are uncertain but are likely, at least in part, a consequence of the extent of inhibition of the tissue ACE activity in the kidney whereby hemodynamic effects appear with less inhibition than is required to elicit structural improvement.[26]

In summary, by exploring the dose-response relationship of RAS blockade in an experimental model of Alport syndrome, the present study shows that ramipril 1 mg/kg/day may be sufficient for examining additive effects on kidney function while 3 mg/kg/day is required in order to assess the effects of add-on therapy on kidney structure as well.

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Ethical considerations

All studies were approved by the St Michael's Hospital Animal Ethics Committee in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publ. no. 85-23, revised 1996)

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Declaration of conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Data availability

All data will be made available on written reasonable request

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