

**PREEMPTIVE RAMIPRIL THERAPY DELAYS RENAL FAILURE AND REDUCES RENAL  
FIBROSIS IN COL4A3-KNOCKOUT MICE WITH ALPORT SYNDROME**

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## Abstract

Alport syndrome (AS) is a common hereditary cause of end stage renal failure in adolescence due to defects in type IV collagen genes. Molecular genetics allows early diagnosis, however, no preventive strategy can be offered. Using the COL4A3  $-/-$  mouse, an animal model for human AS, we evaluated therapy with ramipril in mice.

122 Alport-mice were treated with 10 mg/kg/day ramipril added to drinking water. Proteinuria, serum-urea and lifespan were monitored. Renal matrix was characterized by immuno-histochemistry, light- and electron microscopy and Western blot.

Compared to control, early therapy starting at 4 weeks of age and continuing to death delayed onset and reduced the extent of proteinuria. Uremia was postponed and lifespan increased by more than 100% ( $p < 0.01$ ). In parallel, decreased deposition of extracellular matrix and lessened fibrosis as well as reduced amounts of TGF $\beta$ 1 could be demonstrated. Late therapy starting at 7 weeks decreased proteinuria, however, lifespan did not increase significantly.

Reduced amounts of TGF $\beta$  and fibrosis indicate an antifibrotic effect of ramipril. For the first time, a nephroprotective potential of ACE-inhibitors in a non-hypertensive, non-inflammatory animal model was demonstrated. As ACE-inhibitors also have an anti-proteinuric effect in children with AS, the potential of ramipril to delay renal failure in humans is strongly suggestive. This effect in the mouse is enhanced by initiation of therapy during pre-symptomatic disease. Therefore, early diagnosis and preemptive treatment are crucial in humans.

## Introduction

Alport syndrome (AS) is a hereditary nephropathy characterized by a family history of hematuria and proteinuria, progressive renal failure, sensorineural deafness and typical ocular changes [1, 2]. The disease is caused by mutations in type IV collagen genes, which code for a major constituent of basement membranes. While the  $\alpha1/\alpha2$  (IV) chains are ubiquitous in basement membranes, the  $\alpha3$ (IV)-,  $\alpha4$ (IV)- and  $\alpha5$ (IV)-chains show a restricted distribution and are specifically expressed in the glomerulus, inner ear, and eye [3]. Mutations of one COL4A3, A4 or A5-gene not only alter or abolish expression of the corresponding  $\alpha$ (IV) chain, but also lead to an abnormal basement membrane with decrease or absence of the  $\alpha3$ (IV)-,  $\alpha4$ (IV)- and  $\alpha5$ (IV) chain. How this COL4-mutations initiate the progressive nephritis, renal scarring and subsequent renal failure in AS is not well understood [3, 4, 5, 6].

To date, more than 300 mutations have been described in the COL4A3-5 genes [9, 15], allowing early diagnosis prenatally or in asymptomatic children. However, despite of diagnosis decades before end stage renal failure (ESRF) develops, no therapy can be offered to delay or even prevent renal failure. While studying about 400 families with AS in Germany, Switzerland and Austria, we found increasing evidence, that medical treatment with ACE-inhibitors of children with nephrotic syndrome due to AS without hypertension may lead to a strong reduction of proteinuria and possibly also delay renal failure. However, small family numbers and the 15-30 year long course of AS make it necessary to have a suitable animal model to evaluate the benefit of ACE-inhibitors on renal failure in AS.

The aim of the study was to evaluate the effect of the blockade of the renin-angiotensin-system in a suitable animal model. The COL4A3 knockout mouse was used [7], whose phenotype was found to be very similar to human AS [7, 8]. The effect of ramipril on lifespan,

renal function, proteinuria and renal pathology was determined. In order to find the optimal beginning and duration of medical therapy, different therapeutic regimes were evaluated, including an early versus a late start of therapy, and secondly long versus short duration of therapy.

## Methods

*Genotyping:* Heterozygous COL4A3 knockout mice (Jackson Lab, Maine, USA) were crossbred. Genotyping was carried out by PCR as described before [7, 8]. PCR was repeated in all homozygous animals to exclude artifacts. Tail biopsy, DNA extraction and PCR were repeated in all animals living for more than 12 weeks to ensure the genotype.

*Clinical chemistry and proteinuria:* All procedures and treatment protocols for the mice were previously approved by local German authorities (AZ K 17, 7/00) and supervised by veterinarians. Animals were divided into groups of 4 to 8 mice. Proteinuria and serum-urea were monitored every other week starting at week 4 (figures 2 and 3). Urine was sampled by placing mice in metabolic cages. 3  $\mu$ l of urine were used for microelectrophoresis on a gradient polyacrylamide gel, a semiquantitative technique used in human nephrology to qualify (by molecular weight from 10 to 1000 kDa) and quantify proteinuria (protein diagnostic laboratory, Department of Internal Medicine I, Merheim Medical Center, Cologne, Germany). The major advantage of this technique compared to SDS-PAGE of urinary proteins is that only microliters of urine are necessary for reliable results. Serum-urea-levels were analyzed in 250-1000  $\mu$ l blood from sacrificed animals, placed in heparin-covered capillaries (generally used in human newborns) on a Hitachi 917 Automatic Analyzer (Boehringer Mannheim). Animals were weighed every other week, lifespan was monitored daily.

*Medical therapy:* Following the recommendations of the manufacturer (Aventis GmbH, Germany), ramipril was added to the drinking water, stored at 4<sup>0</sup>C and replaced twice a week. Ramipril remains stable in water for more than 4 days at room temperature [20]. Daily fluid

intake was measured in animals of different sex and age for a two months period. This method ensured, that a daily dose of 10 mg/kg/day ramipril was applied via drinking water during the whole experiment. This dosage in mice is well below the toxic range and equivalent to the maximum therapeutic dose of 10 mg per day in humans [20].

*Animal Groups:* Mice were been bred on a 129/SvJ genetic background to reduce individual differences. Where possible animals from the same litter were allotted to different treatment-groups again to minimize any possible genetic variation. Heterozygous and wildtype mice from the same litter were kept in the same cages to serve as controls. A total number of 122 homozygous COL4A3 null mice were divided into four groups for medication:

- (I) untreated animals, (n=56),
- (II) late therapy, mice with proteinuria >3g/l, starting at week 7 (n=18),
- (III) early, short therapy starting at the 4th week of life (before onset of proteinuria) until week 10 (when uremia develops) (n=24),
- (IV) early, long therapy starting at the 4th week of life (before onset of proteinuria) until death (n=24).

*Statistics:* Data were analyzed by Log Rank statistic (survival analysis, see Box-Plot figure 1) and two-way ANOVA.

*Light and electron microscopy:* Mice were transcardially perfused with a solution of 2% paraformaldehyde and 2% glutardialdehyde buffered with 0.1 M sodium cacodylate, pH 7.3. Kidneys were immediately removed and immersion-fixed for 1 to 3 days. For epoxy resin embedding, external renal cortex was washed extensively with 0.1 M sodium cacodylate and

postfixed in 1% buffered osmium tetroxide for 4 hours. Tissue was dehydrated in graded ethanol including an uranyl acetate *en bloc* staining step overnight in 70% ethanol. Before infiltration with Araldite Cy 212 epoxy resin (Serva, Heidelberg, Germany), propylene oxide was used as intermedium. Tissue blocks were cured for 60 h at 60°C. Semithin (500 nm) and thin (60 nm) sections were taken on a Reichert Ultracut UCT ultramicrotome. Glomeruli were identified in semithin sections stained with methylene blue and in thin sections stained with uranyl acetate and lead citrate. A Zeiss Axiophot microscope (Göttingen, Germany) for light microscopy and a Zeiss EM 902 microscope for electron microscopy were used for histological documentation.

*Immuno-histochemistry:* Animals were sacrificed and kidneys for cryo-sections embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), stored at  $-80^{\circ}\text{C}$  for subsequent 6-7  $\mu\text{m}$  sections on a Leica Kryotom CM3050 (Bensheim, Germany). For paraffin-sections, kidneys were fixated in 4% paraformaldehyde plus 0.1 M phosphate buffered saline (PBS) and vacuum-embedded in a Shandon Citadel1000 (Pittsburgh, USA). Paraffin embedded kidneys were sectioned at 4-5  $\mu\text{m}$  on a Microm HM355S (Walldorf, Germany). Sections were washed three times in PBS for 5 min at room temperature and blocked by incubation with 5% bovine serum albumin in PBS for 30 min at room temperature. Sections were incubated overnight at  $4^{\circ}\text{C}$  with the primary antibodies rabbit anti-EHS-laminin (1:1000, gift from M. Paulsson, Cologne, Germany), and goat anti-fibronectin (1:100, St. Cruz, Heidelberg, Germany). As negative control, slides were incubated with control immunoglobulin. The sections were then washed three times in a solution of PBS and 1.9% NaCl. Subsequently, secondary antibodies labeled with Cy3 as fluorescent dye (goat anti-rabbit IgG and goat anti-mouse IgG from Jackson ImmunoReagents) were added for 1 h, followed by the washing procedure described above.

*Western-Blot:* Aliquots of tissue extracts from 2-3 kidneys of different animals (30  $\mu$ g protein) were dissolved in SDS-sample buffer, separated by electrophoresis in a SDS-polyacrylamide gel (12.5%) under reducing conditions, transferred to a nitrocellulose membrane, and blocked for 60 min at room temperature with 5 % milk-powder in a 0.2 M Tris-HCl buffer, pH 7.6, containing 0.1 % Tween 20 solution (Fisher Scientific) (TBST buffer). Mouse anti-TGF $\beta$ 1 (R&D Systems, Minneapolis, USA) as primary antibody was diluted in TBST and then added to the membrane and allowed to incubate for 60 min at room temperature. The membrane was washed three times with TBST and then incubated for 60 min with secondary antibody conjugated with HRP (Dako). The membrane was washed as above, and the blot was developed using chemoluminescence. Each Western Blot was repeated three times.

## Results

### *Ramipril extends lifespan of COL4A3 -/- mice.*

A total number of 552 animals, 22 % of them being COL4A3 -/-, were generated within a 9 month period. All animals were from a SvJ/129 background to minimize inter-individual genetic differences. Life expectancy was continuously documented during the experiment (figure 1). No animals were lost due to infections or adverse effects of therapy during this trial and no obvious side effects were noted. No signs of severe hypertension such as heart-hypertrophy, increased septum-size or media-sclerosis were found in any animals.

The lifespan of untreated COL4A3 -/- mice (group I) was  $71 \pm 5.7$  days. While the life expectancy of mice in group II (late therapy) did not increase significantly ( $75 \pm 6.5$  days, confidence interval (CI): minus 2 until +9 days) those in group III (early short term therapy until week 10) had a significant increase in lifespan of almost 50% ( $104 \pm 13$  days,  $p < 0.01$ , 99% CI: 23-43 days, figure 1). Mice in group IV (starting therapy early and continued until death) showed an even better response with their lifespan more than doubled to  $150 \pm 21$  days ( $p < 0.01$ , 99% CI: 64-92 days). All treated animals showed a normal behavior and improved weight gain when compared to untreated mice (data not shown). Irrespective of the treatment regime COL4A3 -/- mice could not be distinguished from healthy control animals until uremic symptoms developed a few days before death.

### *Proteinuria and uremia are delayed in COL4A3 -/- mice by ramipril therapy*

Untreated COL4A3 -/- developed proteinuria at about 6 weeks of age, proceeding to typical “nephrotic syndrome” by week 8, with edema, hypercholesterinemia ( $131 \pm 15$  versus  $97 \pm 12$

mmol/l in wildtype-controls;  $p < 0.01$ , data not shown). By this age the proteinuria having reached  $>5\text{g/l}$  (figure 2) and increasing even higher to  $>10\text{g/l}$  prior death from ESRF.

Ramipril reduced the level of proteinuria and slowed down its increase in all groups. Group II (late therapy) while having no effect upon lifespan did reduce proteinuria by over 70% from about  $12\text{g/day}$  to about  $3.5\text{g/day}$  (compared to untreated COL4A3  $-/-$  mice in week 9). Early therapy (group III and IV) delayed onset of proteinuria by two weeks and reduced its amount by almost 80% from about  $12\text{g/day}$  to less than  $3\text{g/day}$  in week 9. There were no significant differences in the reduction of proteinuria in the early versus the late therapy group in week 10 and 11. A slight increase of proteinuria and of creatinine-clearance (data not shown) were noted in week 11 in the early short therapy group (group III) one week after discontinuation of ramipril. This might be caused by an increase in the intraglomerular pressure after withdrawal of the drug.

In untreated mice serum urea started to rise above  $50\text{ mg/dl}$  in week 7, indicating deterioration of renal function (figure 3). Mice became uremic (urea  $247 \pm 27\text{ mg/dl}$  in week 9), ate and drank less and died from hyperkalemia due to ESRF soon after. Late therapy with ramipril, starting at week 7 (group II), significantly reduced urea levels to  $169 \pm 19\text{ mg/dl}$  in week 9 ( $p < 0.05$ ) while early therapy (group III and IV) delayed any elevation of urea by 3 weeks to week 10. The early therapy also slowed down the speed of deterioration of renal function as could be shown by urea levels in week 12 (figure 3, early short therapy  $102 \pm 30\text{ mg/dl}$ , early long therapy  $78 \pm 19\text{ mg/dl}$ ;  $p < 0.01$ ).

*Glomerular basement membrane changes occur in COL4A3  $-/-$  mice despite therapy.*

Typical for AS is the characteristic thickening and splitting of the GBM. Electron microscopy of COL4A3  $-/-$  mice showed these changes to be present irrespective of the therapy regime (figures

4e and i) with no obvious modification in the pathological structure of the GBM between either treated or untreated COL4A3 <sup>-/-</sup> animals. However, abnormal intracellular amounts of fibrillar collagens and a complete loss of the podocyte foot-processes in untreated animals could be observed at lower magnification (figure 4f). These changes appeared to be improved by early ramipril therapy as foot-processes and the slit-membrane could still be found at week 7 (figures 4I and j).

*Ramipril reduces abnormal deposition of the renal extracellular matrix in COL4A3 <sup>-/-</sup> mice leading to fibrosis and renal failure.*

Light microscopy (figure 4) showed periglomerular and interstitial fibrosis in untreated animals (figure 4g) with marked glomerular hypercellularity and thickening of Bowman's capsule. Tubular cells contained numerous intracellular vesicles, due to reabsorption of urinary proteins which occurs in severe proteinuria. Changes proceed to glomerulosclerosis and –fibrosis with a complete loss of glomerular function and nephrons, leading to end stage renal failure in week 10 (figure 4h).

Fibrotic changes were far less severe in the early therapy group in week 7 (figure 4k) and the differences between untreated (figure 4h) and treated (figure 4I) animals were even more striking by week 10, with ramipril treatment leading to glomerular architecture and marked less fibrosis.

Immuno-histochemistry confirmed changes in the extracellular matrix (figure 5): While wildtype mice showed scant staining for fibronectin (figure 5a) this was markedly increased in the peri-glomerular matrix of untreated COL4A3 <sup>-/-</sup> mice, a typical finding seen in severe glomerulosclerosis (figure 5b). The staining for fibronectin was much reduced in treated animals being only slightly stronger than in healthy controls (figures 5c and a).

Increased deposition of extracellular matrix was also demonstrated by staining with a polyclonal antibody reacting with the three subunits in laminin 1 (laminins  $\alpha 1$ ,  $\beta 1$ ,  $\gamma 1$ ). Healthy controls showed a thin tubular and glomerular basement membrane (GBM) (figure 5*d*) with no intertubular deposition of laminin when viewed at high magnification. In contrast, untreated animals showed a severely increased deposition of extracellular matrix (figure 5*e*), laminin staining being present intra- and periglomerular, as well as in the intertubular regions. Localised shrinkage of tubular lumen was noted, indicating loss of function of different nephrons. Early treatment with ramipril resulted in an increased signal of the GBM of COL4A3  $-/-$  mice (figure 5*f*) when compared to healthy controls (figure 5*d*), probably due to the ultra-structural changes documented above (electron microscopy in figure 4*i*). However, compared to untreated COL4A3  $-/-$  mice, periglomerular and intertubular signal was markedly decreased with an almost normal staining in the intertubular space and a preserved tubular lumen, suggesting a preserved function of nephrons in treated mice.

*Early ramipril therapy leads to reduced TGF $\beta$ 1 expression in COL4A3  $-/-$  mice.*

One major effect of ramipril is believed to be the downregulation of TGF $\beta$  which results in a reduction in fibrosis. Total protein was extracted and 30  $\mu$ g aliquots (as shown by BCA protein assay (Pierce)) were separated by PAGE, and immunoblotted with an antibody against TGF $\beta$ 1. Figure 6 shows the TGF $\beta$ 1 signal in pooled kidneys of treated versus untreated COL4A3  $-/-$  mice: Compared to untreated COL4A3  $-/-$  mice (lanes 5 and 6), early ramipril-therapy (lanes 3 and 4) causes a strong downregulation of TGF $\beta$ 1. This effect is much reduced in the late therapy group (lane 1). Compared to early long therapy in week 12 (lane 7), levels of TGF $\beta$ 1 in the early short therapy group start to rise 2 weeks after discontinuation of treatment.

## Discussion

The major goal of this study was to resolve a clinical dilemma occurring in children with AS, of being capable of an early diagnosis while having no proven therapeutic options. Due to small family numbers, the variability in the genetic mutations in the COL4A genes, and the long course of disease where one has to wait 20-30 years to evaluate the benefit of any therapeutic intervention, there is no reliable clinical data available. In assess the potential benefit of medical therapy on renal changes in AS requires an appropriate animal model.

The COL4A3  $-/-$  mouse was shown to be a suitable animal model of human AS. In man, over 300 different mutations are known in either  $\alpha 3$ ,  $\alpha 4$  or  $\alpha 5$  chains of type IV collagen and result in differences in the severity of the AS phenotype [9]. Here all the untreated animals showed a very similar temporal onset of uremia and proteinuria resulting in death between 10 and 11 weeks of age. These highly reproducible changes are due to the common null mutation of the  $\alpha 3$  (IV) chain as well as the identical 129 genetic background. Further the use of animals maintained under specific pathogen free housing conditions reduced the likelihood of intercurrent infections altering the course of the renal disease. The model also allows a high number of “patients” leading to statistically significant data. Thus clear clinical endpoints could be obtained and evaluated further by immuno-histochemistry, light-, and electron microscopy and immunoblot analysis of renal tissue. Together this allowed us to assess the effect of ramipril in this model for human AS.

The COL4A3 knockout mouse is the first non-hypertensive, non-inflammatory animal model of chronic progressive renal disease. A nephroprotective and antifibrotic effect of ramipril was clearly demonstrated: Lifespan until death from uremia – the clearest and most important end point – was prolonged by more than 100% in early long therapy, an impressive effect.

Early starting life-long therapy had the greatest effect on lifespan, but the reason for this is open to conjecture. One major effect of ACE-inhibitors such as ramipril is their ability to lower blood pressure. However, a reduction in blood pressure has never been shown to have a beneficial effect for humans suffering from AS and indeed during the first 10-15 years, hypertension does not appear to play a major part in the pathogenesis of the renal failure [16]. Similarly in this mouse model there were no signs of severe hypertension. Several other points in our study indicate, that the antihypertensive effect of ramipril is not crucial in prevention of renal failure: In the late therapy group of COL4A3  $-/-$  mice which received ramipril from week 7 and when one might have expected signs of renal hypertension to develop there was no improvement in lifespan in any statistical significant manner. Further animals stopping treatment at week 10, after the elevation of blood urea levels, had a markedly improved lifespan, though one would have expected severe hypertension upon withdrawal of the antihypertensive leading to a rapid deterioration in renal function. These results are in agreement with the findings that ACE-inhibitors have nephroprotective effects independent of lowering systemic blood pressure for instance in dogs with X-linked hereditary nephritis [20].

Urinary proteins have been shown to induce progressive interstitial fibrosis [21] and the known antiproteinuric effects of ACE-inhibitors have been suggested to be nephroprotective. In this study, a strong antiproteinuric effect of ramipril could be demonstrated in all treated mice, however no significant difference in the degree of proteinuria were found in the “late therapy” when compared to “early therapy”-groups even though animals in the later group live twice as long. This indicated that the antiproteinuric effects of ramipril are not crucial in preventing renal fibrosis and renal failure in these mice.

Ramipril has additional effects in preserving renal function in other human renal diseases such as diabetic nephropathy [19] which resembles AS in that matrix alternations are thought to

lead to renal failure 20-25 years after the disease begins. In AS this is due to genetic changes while it appears to be “acquired” in diabetic nephropathy. ACE-inhibitors are known to block conversion of Angiotensin I to Angiotensin II, a renal growth factor which activates fibroblasts leading to an increased synthesis of extracellular matrix proteins. Angiotensin II has also shown to be profibrotic, behaving as a cytokine, activating mononuclear cells and increasing proinflammatory mediators [10], as well as regulating matrix degradation. Some of these effects are mediated via the TGF $\beta$ -pathway which has been shown important in the renal fibrosis in AS [11, 12], suggesting that blockade of the renin-angiotensin-system might be a therapeutic option in children with AS. Our data show that TGF $\beta$  levels are increased in the COL4A3<sup>-/-</sup> mice. Early intervention with ramipril results in a reduced expression, however, TGF $\beta$  rapidly increases after the discontinuation of early ramipril-therapy. This paralleled the deterioration in renal function. These results emphasize the role of TGF $\beta$  in progression of AS and outline the importance of continuous application of ramipril in humans in order to delay renal fibrosis.

Presumably ramipril's regulation of TGF $\beta$  and matrix-metalloproteinase inhibitors (MMPs) [10], that trigger fibrosis and matrix degradation, leads to the marked reduction of periglomerular and tubulointerstitial fibrosis (figure 5). Application of MMP-inhibitors in early course of the disease in COL4A3<sup>-/-</sup> mice produced similar nephro-protective effects as described here [13]. Further, ramipril as with the MMP-inhibitors lost its profound nephroprotective effect, once renal fibrosis and deterioration of renal function developed (in COL4A3<sup>-/-</sup> mice 7 weeks or older). Ramipril did not improve structural damage of the GBM, caused by the loss of the  $\alpha$ 3(IV),  $\alpha$ 4(IV) and  $\alpha$ 5(IV)-chain and persistence of the  $\alpha$ 1/ $\alpha$ 2(IV)-chain. Previous studies showed some evidence, that abnormal composition of the GBM leads to secondary events resulting in renal fibrosis [11, 12]. Ramipril influences the altered matrix-cell-interaction of the glomerulus,

preventing tubulointerstitial fibrosis as well (figure 5). Indeed, integrin  $\alpha 1$ / COL4A3 double-mutant mice have been shown to have a slower progression of renal disease and a delay in ESRF [12]. Presumably this is due to a lack of the interaction of Integrin receptors with the abnormal basement membrane. Similar effects might be involved in the present study.

Most children with nephrotic syndrome of different causes are treated with ACE-inhibitors. Because of the level of experience with this drug and its few side effects, numerous children of our Alport-families already receive ACE-inhibitors. This therapy showed antiproteinuric effects in all children, however, one will have to wait 20 years (or if successful even longer) until patients develop renal failure to demonstrate a similar beneficial effect to that seen in mice.

Cyclosporine A is the only drug, previously shown to reduce proteinuria in children with AS [14, 17]. However, beneficial effects on preserving renal function have not yet been demonstrated. In addition, treating Alport-children with this immunosuppressive, potentially nephrotoxic drug has many drawbacks, hindering us to use cyclosporine A in any of our 400 Alport families. For the first time, an alternative to cyclosporine A with much less dangerous side effects and one which has been proved to have nephroprotective and antifibrotic effects leading to a delay in renal failure has been analyzed in an adequate animal model for human AS.

Ramipril is estimated to delay renal failure in humans by more than 20 years. It did not improve renal outcome in our mouse model once fibrosis and deterioration of renal function developed suggesting the importance of early molecular-genetic diagnosis. As ramipril's beneficial effect was strongly enhanced by initiation of therapy early during pre-symptomatic disease, preemptive treatment before onset of proteinuria would appear to be crucial in humans with AS. Genetic counseling should be offered to all children with hematuria in order to start early pre-symptomatic therapy as soon as the diagnosis of AS is made.

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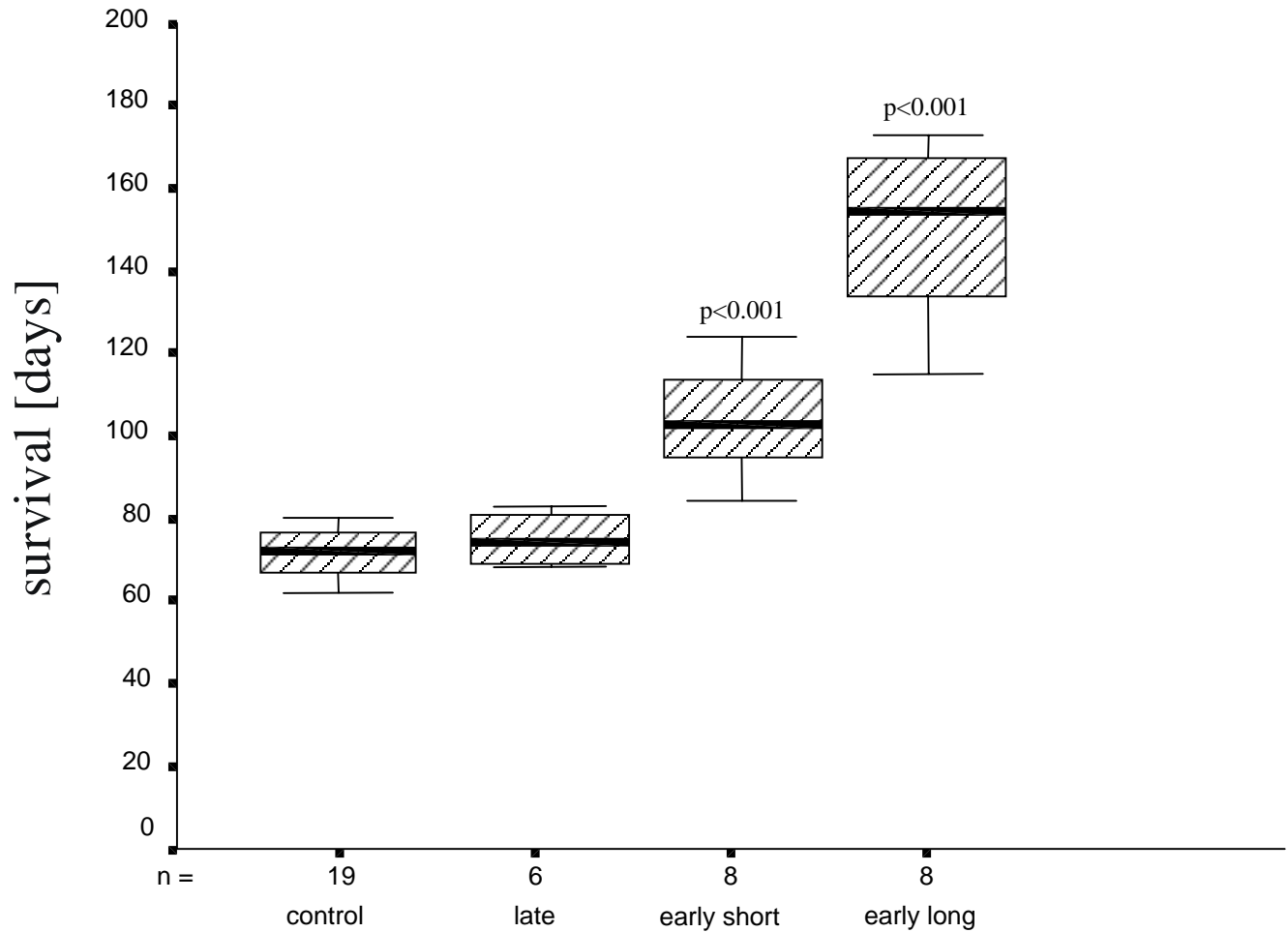


Fig. 1: Box-Plot analysis of lifespan of ramipril treated animals compared to control. Lifespan until death from renal failure increased significantly by early short treatment (group III) until the 10th week of age from  $71 \pm 5.8$  to  $104 \pm 13.3$  days ( $p < 0.001$ ). When therapy was continued beyond the 10th week (group IV), animals lived twice as long ( $150 \pm 20.7$  days;  $p < 0.001$ ) as untreated COL4A3-knockouts. Late therapy (group II) did not increase lifespan significantly ( $75 \pm 6.5$  days).

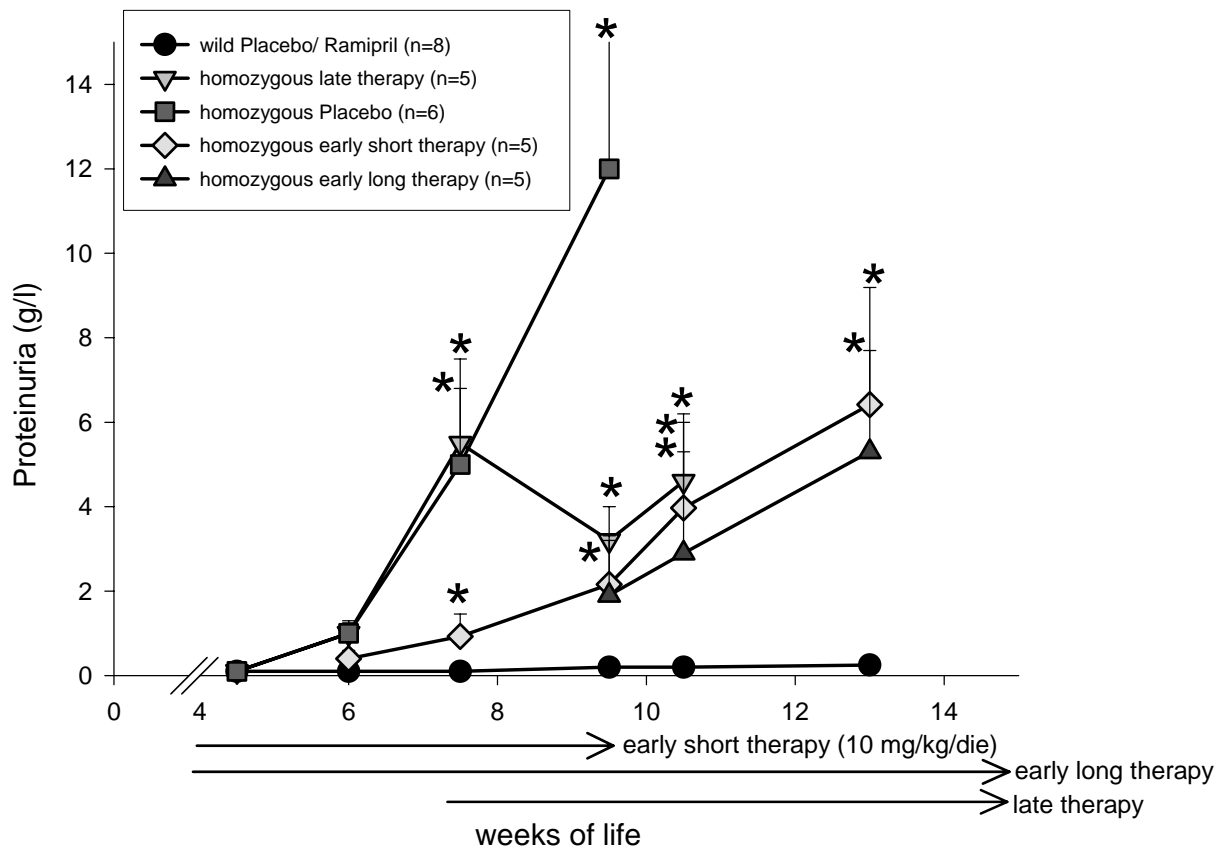


Fig. 2: Proteinuria was measured by urine-protein-microelectrophoresis every other week. Compared to untreated COL4A3 <sup>-/-</sup> mice, early therapy delayed onset of proteinuria by 2 weeks and reduced its increase and its maximal amount by >80% in week 9. When ramipril therapy was continued after the 10th week of life, no additional beneficial effect on reducing the amount of proteinuria was found. However, discontinuation of medication in the early short therapy group lead to a slight increase of proteinuria due to an elevated intraglomerular pressure. Late therapy was able to reduce proteinuria to almost the same degree than early therapy did.

(\* = p<0.05)

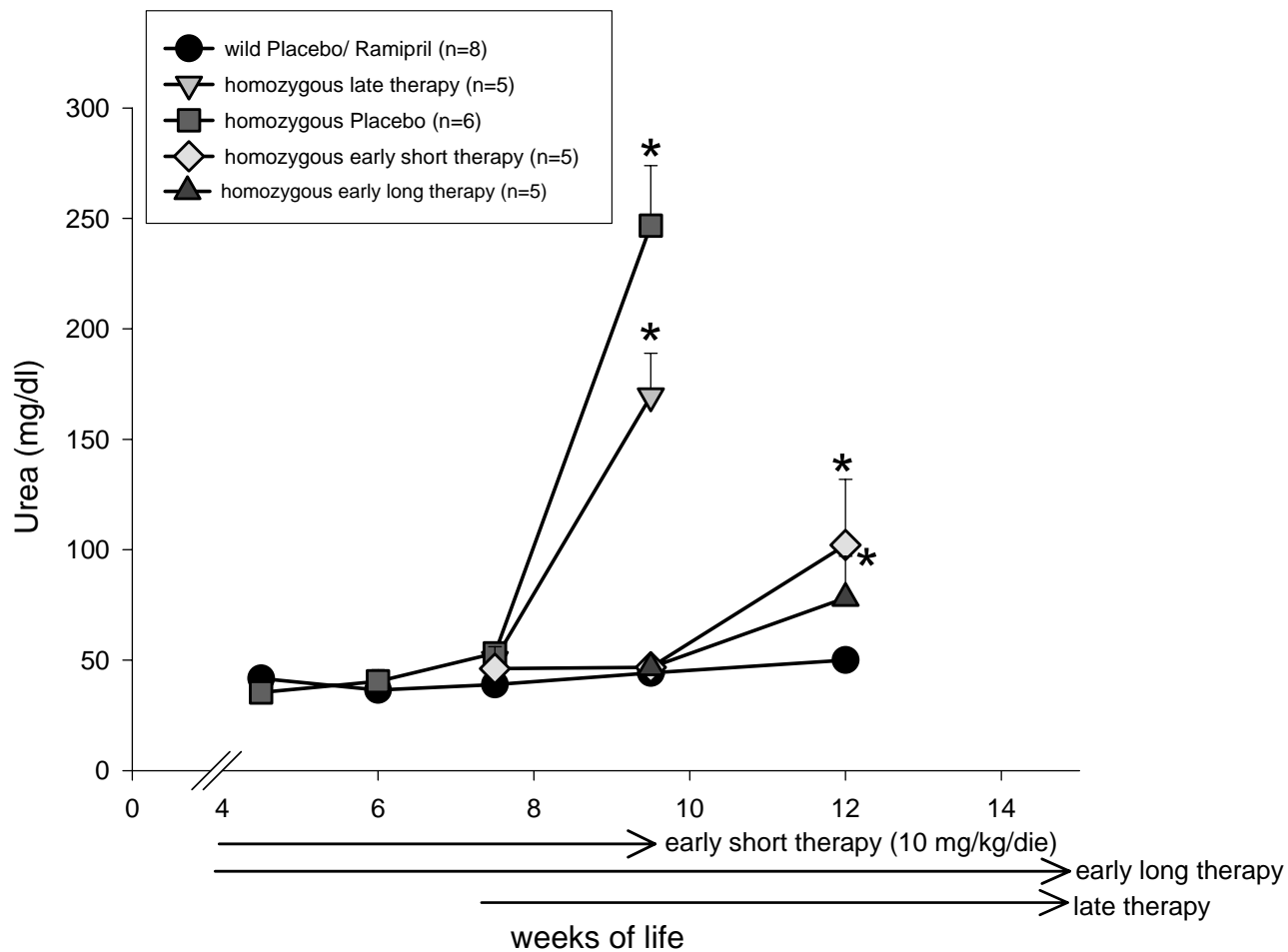


Fig. 3: Serum urea levels were measured every other week. Elevation of urea above normal range (>50 mg/dl) was postponed by early short and early long therapy by 3 weeks (7.5 vs. 10.5 weeks,  $p < 0.01$ ). Late therapy did not delay elevation of urea.

(\* =  $p < 0.05$ )

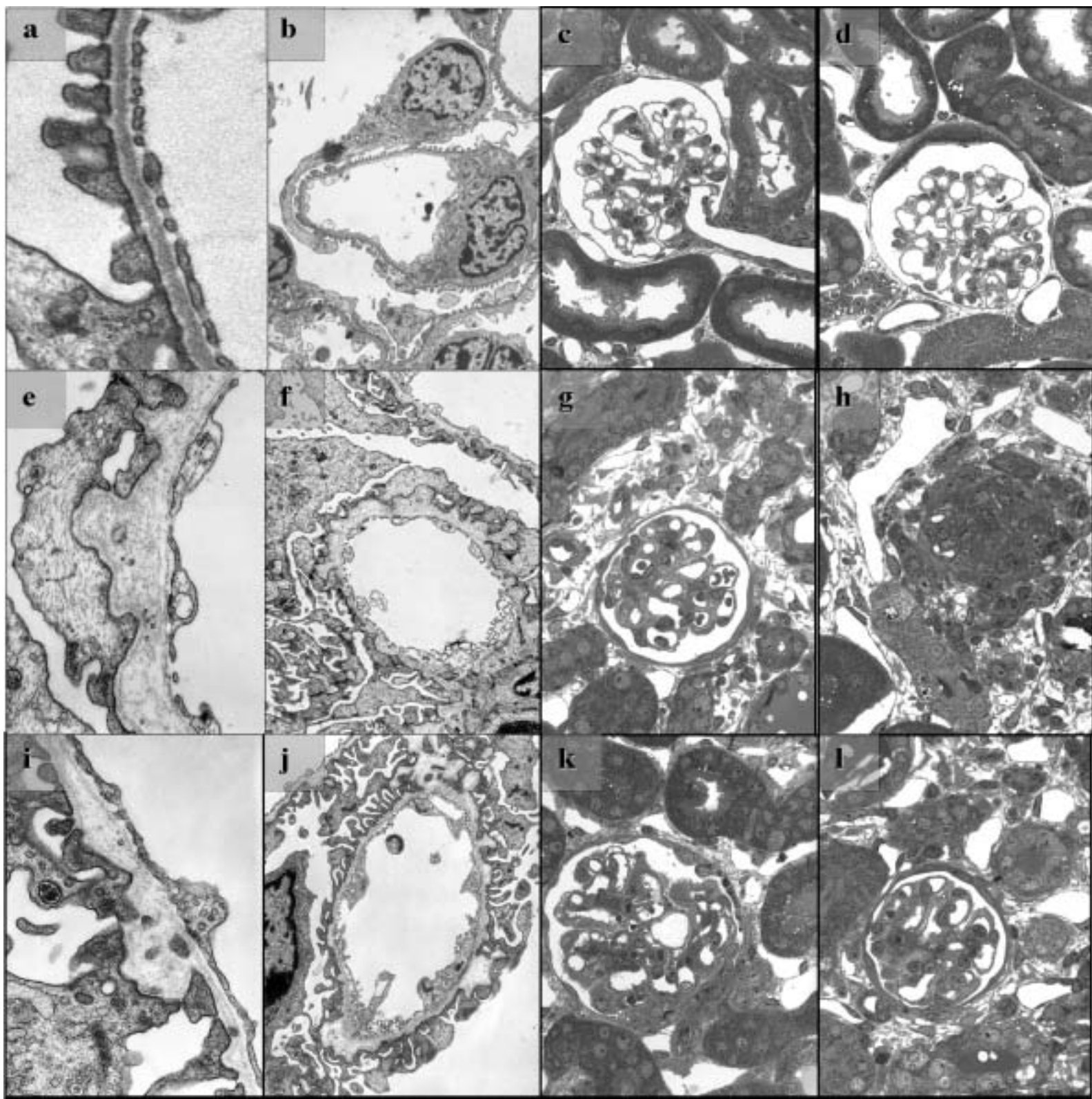


Fig. 4 Representative figures from wildtype controls (upper row, **a-d**), untreated COL4A3  $-/-$  (middle row, **e-h**), and treated COL4A3  $-/-$  (lower row, **i-l**).

*Electron microscopy:* All mice at 7 weeks of age. Healthy controls show a typical trilaminar structure of the glomerular basement membrane, consisting of two laminae rarae and one lamina densa. Normal podocyte foot-processes are noted with the split-membrane in between (**a, b**). In contrast, both treated (**i**) and untreated (**e**) COL4A3  $-/-$  mice show characteristic thickening and splitting of the GBM (magnification: 15,000- to 20,000-fold). Lower magnification (5,000-7,000-fold) demonstrates a complete loss of the podocyte foot-processes, a marked hypercellularity and intracellular masses of fibrillar collagens (**f**). These changes appear to be slightly improved by early therapy in week 7 (**j**), as some foot-processes with a normal looking split membrane are seen (**i**).

*Light microscopy:* 500 nm kidney-sections of untreated animals (**g**) show periglomerular and tubulointerstitial fibrosis in week 7, leading to marked general fibrosis and glomerulosclerosis, that cause end stage renal failure in week 10 (**h**). In the treated group, less fibrotic changes are noted in week 7 (**k**). Delayed fibrosis leads to a preserved architecture of the glomerulus in week 10 (**l**) (healthy controls: **c, d**; magnification: 600-800 fold).

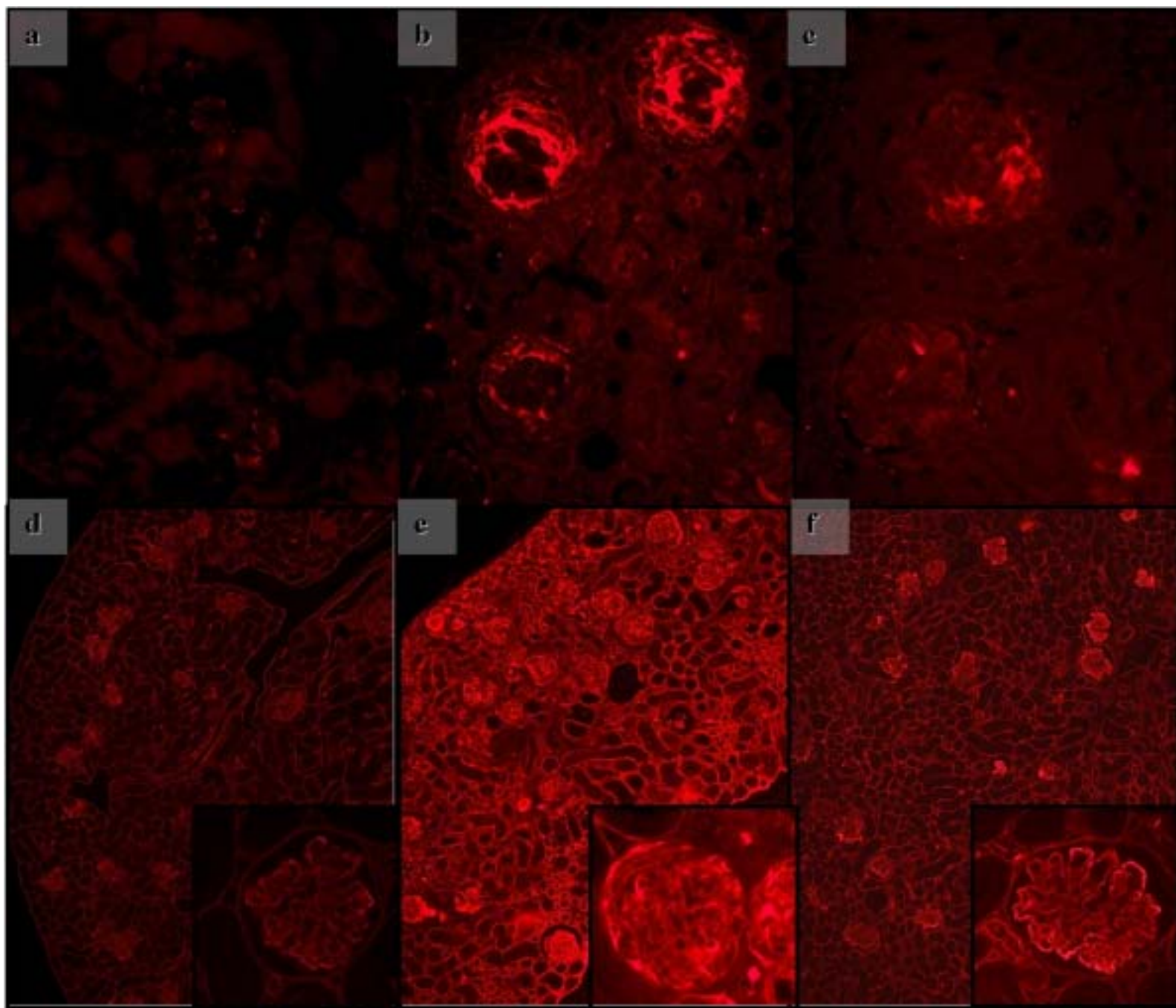
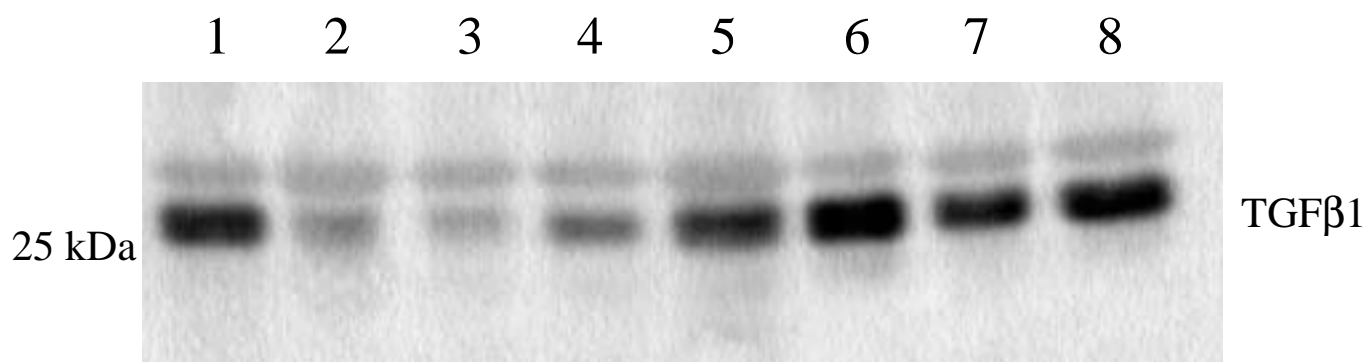


Fig. 5 Representative figures from wildtype controls (left side, **a** and **d**), untreated COL4A3 <sup>-/-</sup> mice (middle, **b** and **e**) and treated COL4A3 <sup>-/-</sup> mice (right side, **c** and **f**).

*Immuno-histochemistry:* All 3 glomeruli stained for fibronectin show severe glomerulosclerosis in untreated mice (**b**, healthy control: **a**). This is much less apparent in the 3 glomeruli shown of treated animals (**c**). Deposition of extracellular matrix is demonstrated by staining for laminin 1 in **d**, **e** and **f** in an overview and a high magnification of one glomerulus. The overview allows an objective, representative impression on renal damage and fibrosis. Differences in amounts of extracellular matrix are so clear in **d**, **e** and **f** that no subjective grading of fibrosis was done. Compared to healthy controls (**d**), untreated animals (**e**) show a severely increased deposition of extracellular matrix in week 10. This deposition of extracellular matrix is markedly decreased in the treated group (**f**). In correspondence to the thick and splitted GBM in both treated and untreated animals in electron microscopy, high magnification of one glomerulus in the treated group (**f**) shows an abnormally thick and split basement membrane (healthy control: **d**).



1	homozygous,	10 weeks,	late therapy
2	wildtype,	10 weeks,	control
3	homozygous,	7.5 weeks,	early long therapy
4	homozygous,	10 weeks,	early long therapy
5	homozygous,	7.5 weeks,	control
6	homozygous,	10 weeks,	control
7	homozygous,	12 weeks,	early long therapy
8	homozygous,	12 weeks,	early short therapy

Fig. 6 Western Blot for TGF $\beta$ 1 (25 kDa) in treated and untreated Alport-mice. Compared to untreated COL4A3  $-/-$  mice (lanes 5 and 6), early ramipril therapy (lanes 3 and 4) causes a strong downregulation of TGF $\beta$ 1. This effect is much less apparent in the “late therapy” group (lane 1). Compared to “early long therapy” in week 12 (lane 7), levels of TGF $\beta$ 1 in the “early short therapy” group again start to rise two weeks after discontinuation of therapy, suggesting that TGF $\beta$  may be involved in the fibrotic process.