Exploring the molecular basis of ramipril-induced nephroprotection in Alport syndrome

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Background

- Alport syndrome is an inherited condition characterised by progressive kidney disease, sensorineural hearing loss, and ocular abnormalities. Alport syndrome is caused by pathogenic variants in either *COL4A3*, *COL4A4* and *COL4A5*, the genes encoding type IV collagen α3/α4/α5 chains.
- Renin-angiotensin system inhibitors (RAASi) such as angiotensin-converting enzyme Inhibitors (ACEi) play a central role in the treatment of chronic kidney disease (CKD), and they have been proven to prolong kidney survival and reduce urinary protein excretion in Alport syndrome.
- The protective effect of RAASi has been explained by the reduction in intraglomerular pressure and the inhibition of inflammation and subsequent fibrosis. However, the precise molecular mechanisms of ACEi protection in Alport kidneys are yet to be elucidated.
- We used mass spectrometry-based proteomics to identify molecular pathways and components affected by ramipril treatment in *Col4a5* knockout (Alport) mice.

Methods

- The ACEi ramipril was administered orally (n=10 each) to a mouse model of Alport syndrome (*Col4a5* knockout). Treatment was initiated at five weeks (early treatment group) or ten weeks (late treatment group) and continued until 16 weeks of age.
- We evaluated clinical parameters such as body weight, urine protein, kidney histology and mass spectrometry-based proteomic analysis of isolated kidney glomeruli.

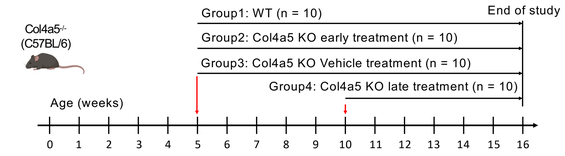


Figure 1. Treatment schedule of the study

Results

① Functional evaluation

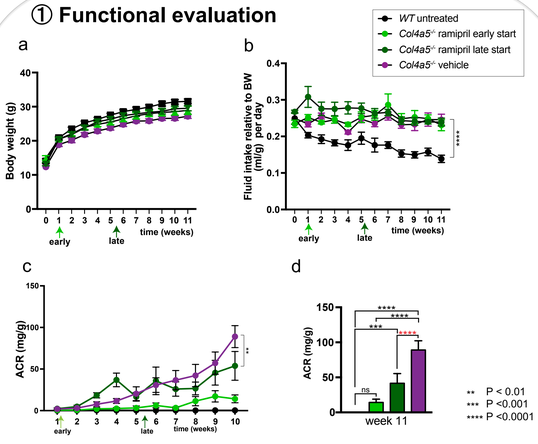


Figure 2. Clinical evaluation of Ramipril treatment.
(a) There was no significant changes in body weight. **(b)** Fluid intake was increased in the Alport mice. **(c)(d)** The urinary albumin creatinine ratio (ACR) was reduced in ramipril treated groups compared to the vehicle treatment group. However, there was no significant difference between early and late ramipril treatment groups.

② Pathological findings

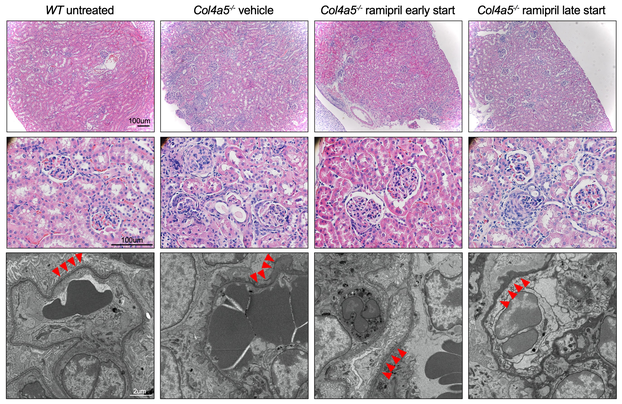
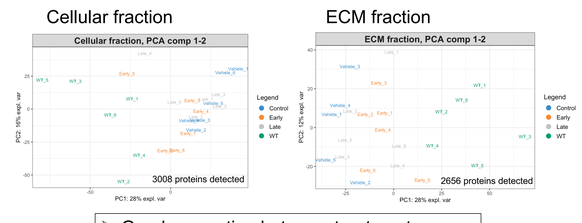


Figure 3. Light microscopic and electron microscopic findings at 16 weeks.
Inflammatory cell infiltration to the interstitial area and glomerular sclerosis were prominent findings in the vehicle and late ramipril groups, while such changes were minor in the early ramipril treatment group. Similarly, the glomerular basement membrane (red triangle) was thicker in the vehicle and late ramipril groups.

③ Mass spectrometry

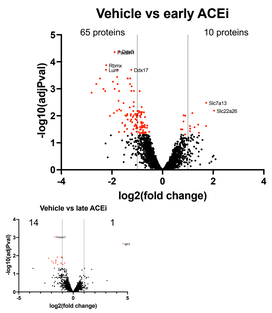
(A) PCA analysis



➢ Good separation between treatment groups

(B) Volcano plot

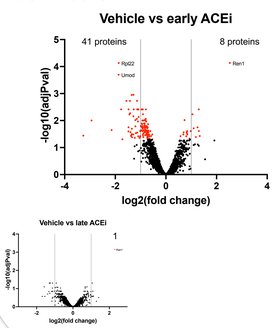
Cellular fraction



up-regulated in vehicle				
Acat1	Decr1	Khsrp	Ptbp3	Thoc2
Actl6a	Eefsec	Loxl2	Rbmx	Thy1
Alyref	Eif2s1	Lum	Rbmxl1	Tnc
C4bp	F13a1	Mup21	Rcc1	Tpm1
Capg	Fam98b	Myh10	Rps27	Umod
Cbx3	Farsb	Myh9	Safb	
Cct3	Fau	Myl6	Serpinh1	
Cct4	Fxr2	Nebl	Sfpq	
Cct5	Hmgn2	Nono	Smc3	
Col15a1	Hnrnpa0	Oas1a	Ssrp1	
Coro2a	Hnrnpa3	Oqn	Surf4	
Dazap1	Hnrnpdl	Pacsin1	Syncrip	
Dcn	Hnrnph3	Pdlim2	Tardbp	
Ddx17	Hnrnpu	Pds5b	Tcp1	
Ddx5	Khdrbs3	Prelp	Thoc1	

in early ramipril
Abcb1a
Ace
Gja5
Ndufs6
Park7
Slc22a13
Slc22a26
Slc3a1
Slc7a13
Tmem27

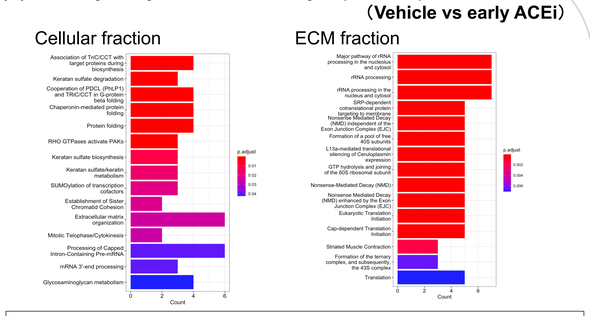
ECM fraction



up-regulated in vehicle		
Alyref	Lemd2	Rps19
Cc2d1a	Lgals3	Rps28
Ckap4	Lum	Rps29
Coro1c	Mat2a	Serpine2
Dhrs4	Mfap5	Snrpb2
Dynll1	Mpeg1	Synpo
Exosc4	Mup2	Tpm1
Exosc5	Myadm	Tpm4
Fau	Myh4	Umod
Fbn2	Ppib	Usp39
Hdgfl3	Pyhin1	Vim
Hmga1	Rcc1	
Hmgn2	Rpl21	
Ifit1	Rpl22	
Kctd12	Rpl36	

in early ramipril
Epx
Gcat
Hapln1
Itih5
Ren1
Slc16a2
Slc22a8
Slc7a13

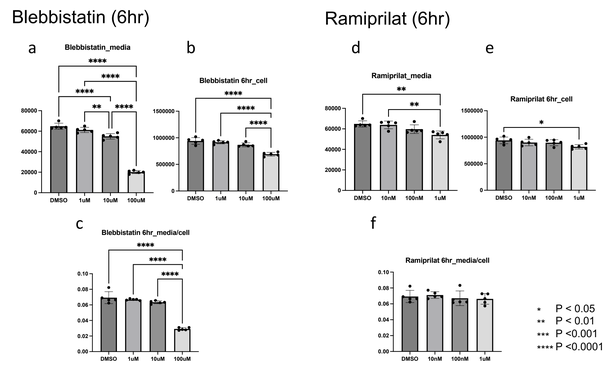
(C) Pathway analysis of differentially expressed proteins (Vehicle vs early ACEi)



➢ Significant enrichment for terms associated with chaperonin-mediated protein folding, extracellular matrix organisation and glycosaminoglycan metabolism

④ in vitro ECM secretion analysis

➢ We investigated the potential effect of non-muscle myosin inhibition (blebbistatin) or ramiprilat (a bioactive metabolite of ramipril) on the secretion of extracellular matrix using human podocytes with HiBiT-tagged COL4A2.



Blebbistatin reduced both COL4A2 secretion into the medium and intracellular accumulation **(a.b)**. The secretion into the medium was most notable **(c)**. In contrast, ramiprilat only reduced type collagen IV secretion into the medium **(d)** but not in intracellular accumulation in podocytes **(e)**. The ratio of extracellular to intracellular signals did not differ between the groups **(f)**.

Discussion

Functional effects

- The early ACEi treatment group had a greater reduction in proteinuria at the end of the study compared with the late-treatment group. This result confirms the recent human RCTs showing that early introduction of ACEi in Alport syndrome may be effective in reducing proteinuria and in preventing the decline of kidney function.

Pathological findings

- Similar to the functional data (urine ACR), pathological findings showed less fibrosis, less inflammatory cell infiltration and less glomerular sclerosis in the early-treatment group than in the late-treatment group. Although overall survival was not observed in this study, clinical and pathological data suggest that earlier ACEi treatment may reduce the development of kidney fibrosis and inflammation and eventually improve kidney prognosis.

Proteomic analysis

- With proteomics we found more changes with early ACEi treatment compared to vehicle treatment. With pathway enrichment analysis, we found significant enrichment for terms associated with chaperonin-mediated protein folding, extracellular matrix organisation and glycosaminoglycan metabolism in early ACEi-treated Furthermore, looking at the differentially expressed proteins individually, we found increased expression of several non-muscle myosin class II proteins (Myh9, Myh10) in the untreated group.
- MYH9 is expressed mainly in podocytes in the kidney and is thought to play a central role in maintaining the actin cytoskeleton, anchoring podocytes to the glomerular basement membrane and stabilising the slit membrane against mechanical stress such as blood flow. MYH9 variants have also been associated with kidney diseases with glomerular basement membrane findings similar to Alport syndrome (Epstein syndrome; OMIM#155100). The elevated expression of Myh9 in the untreated group suggests that this may be a compensatory change for the high intraglomerular pressure in the absence of ACEi treatment.
- Furthermore, inhibition of myosin action in vitro resulted in an inhibition of type IV collagen secretion in podocytes and a concentration-dependent decrease in the ratio to the intracellular signal. These results suggested that myosin signaling may also be involved in extracellular matrix production and transport.

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