ONLINE METHODS

Study design. Eight Wistar rats (Charles River S.p.A, Calco, Italy) and forty-one male MWF rats from our colony[1] were used in this study. The group of Wistar rats (n=8) was studied at 50 weeks of age and used as normal control. MWF rats were divided into four groups. Group 1, MWF 50 (n=8) consisting of untreated animals, was studied at 50 weeks of age. Group 2, MWF 60 (n=13) was left untreated and followed from 50 to 60 weeks of age. Group 3, MWF+LIS 60 (n=13) and group 4, MWF+LOS 60 (n=7) were treated from 50 to 60 weeks of age with the ACE inhibitor lisinopril (80 mg/L in drinking water) or with the angiotensin II receptor antagonist losartan (100 mg/kg body weight in drinking water), respectively. Two additional groups of Wistar rats (n=4 in each group), of 30 weeks of age, have been used to investigate the effect of ACE inhibition on kidney perfusion by Microfil. Twenty-four hour urinary protein excretion was periodically measured during the observation period by Coomassie method using a Cobas Mira auto analyzer (Roche Diagnostic). All animals were housed in a temperature-controlled room regulated with a 12-hours dark/12-hours light cycle and allowed free access to standard diet containing 18% protein by weight and water.

MicroCT imaging of kidney vasculature. At sacrifice, rats were anesthetized with isofluorane. A PE50 catheter was placed in the thoracic aorta, and the vessel was ligated below and above renal arteries. The right kidney was removed and kidney specimens were obtained for further processing for PCR, renal histology and immunohistochemistry analyses. The vena cava was cut to allow free blood outflow and the left kidney was perfused with saline containing heparin (10 U.I./mL). A freshly mixed radio-opaque silicone polymer containing lead chromate (Microfil MV122, Flow Tech, Carver, MA, USA) was then infused, under a constant pressure of about 120

mmHg, until free drain was observed. Renal vein and artery were ligated, the kidney was left *in situ* for 20 min to allow Microfil polymerization and finally removed. After complete polymerization (about 2 hours) the kidney was immersed in 4% formaldehyde buffered with 0.1 M phosphate (pH 7.2) to complete fixation[2] and left at 4°C. After three days, samples were imaged by microCT system (SkyScan 1076, Bruker-microCT, Kontich, Belgium). Scanning was performed using an aluminium filter (0.5 mm) with source voltage of 47 kV, current 200 mA, exposure time 1800 ms and rotational step of 0.4°. Volume images were reconstructed using a back-projection algorithm using NRecon software (Bruker-microCT) at pixel size of 9 μm with 256 gray levels.

Kidney segmentation was obtained by a semi-automatic procedure based on region growing algorithm using open source MITK software (*www.mitk.org*). Quantitative 3D structure analysis was then performed using CTAnalyser software (Bruker-microCT). After image binarization total kidney volume (TK_V) was computed applying the marching cubes algorithm. For vascular structure segmentation, images were thresholded above gray level 130. Total blood vessel volume (TV_V) was calculated on thresholded images with marching cubes algorithm. Vascular volume density (V_V) was calculated as the ratio between TV_V and TK_V. Vessels diameters along the vascular tree were computed using the "sphere-fitting" method reported by Hildebrand et al.[3] This method allows the direct estimation of structure thickness without a model-assumption and this well applies for the analysis of objects with a complex structure as kidney vasculature. Being $\Omega \in \mathbb{R}^3$ the set of all points of the spatial structure and $\underline{p} \in \Omega$ an arbitrary point in this structure, local thickness $\tau(\underline{p})$ is defined as the diameter of the largest sphere which fulfils the conditions of enclosing the point $\underline{p}$ (which is not necessarily the center of the sphere) and of being entirely bounded within the solid surfaces according to the following equation

$$\tau\left(\underline{p}\right) = 2 * \max(\{r \left| \underline{p} \in sph(\underline{x}, r) \subseteq \Omega, \underline{x} \in \Omega\}\right)$$

where $sph(\underline{x}, r)$ is the set of points contained in the sphere of center $\underline{x}$ and radius r. The maximal local thickness is equivalent to the diameter of the largest sphere that completely fits inside the structure

$$\tau_{max} = \max\left(\left\{\tau\left(\underline{p}\right) \left| \underline{p} \in \Omega\right.\right\}\right)$$

The numerical method starts with a "skeletonisation" used to identify medial axes of all vascular structures, then the "sphere-fitting" local thickness measurement is applied to all the voxels lying along this axis. Blood vessels are then classified according to their diameters in three groups, microvessels with diameter lower than 80 μm, microvessels with diameter between 80 and 180 μm and large vessels with diameter over 180 μm. For each group of vessels, the mean diameter is computed and used to calculate the length of an equivalent vessel, having the same mean diameter and volume.

SEM and morphometric analysis. Vascular corrosion casting was performed in selected animals from Wistar 50, MWF 50, MWF 60 and MWF+LIS 60 group (n=3 each) as described previously.[4] After animal preparation described above, the left kidney was first perfused with heparinised saline followed by infusion of 2% paraformaldehyde in phosphate-buffered saline. Thereafter, the polyurethane resin PU4ii (vasQtec, Zurich, Switzerland, diluted with ethylmethylketone at 30% w/v with addition of a blue pigment, resin/hardener ratio, 6:1 by weight) was infused until complete perfusion of the kidney that uniformly stained in blue. The kidney was

excised and maintained at room temperature for 48 hours. After resin curing, soft tissue was macerated in 7.5% KOH for 48 hours at 50°C and the resin cast was thoroughly rinsed with water and freeze-dried.

Casted glomeruli were dissected from the cortex under a stereomicroscope, mounted onto aluminum stubs on an adhesive film, and coated with 10 nm of gold in a sputter-coater (Agar Scientific Ltd, Stansted, England) with a current of 10 mA for 60 s. Low and high power images of microvascular corrosion casts were taken with a Cross-Beam 1540EsB scanning electron microscope (Carl Zeiss GmbH, Oberkochen, Germany). Filament current was set to 2 kV and in-lens detector was used to generate images of secondary electrons. For morphometric analysis images of 75 randomly selected casted glomeruli were acquired for each animal at fixed magnification (703x).

Morphometric analysis was performed on digital images (1024x768 pixels) using ImageJ software (NIH, Bethesda, MD, USA). To estimate mean glomerular volume (V_G), the outline of the glomerular tuft was manually traced on digital images, and its area automatically computed. Under the hypothesis of spherical geometry, the radius and V_G of each glomerulus were then calculated from maximal glomerular area. To quantify the regularity of the shape of the glomerular capillary tuft, the circularity (C_{GC}) of the maximal glomerular area (A) was calculated using a specific ImageJ plugin,

$$C_{GC} = 4\pi \frac{A}{p^2}$$

where p is the perimeter of the glomerular tuft. A circularity value of 1 indicates a perfect circle while a value $\rightarrow$0 indicates elongated shape.

Mean diameter of glomerular capillary segments was estimated using morphometric analysis. An orthogonal grid (7x10 lines, with distance between lines of 55 μm) was superimposed on 50 SEM images of individual glomerular casts. For each image, the mean diameter of capillary segments was estimated using the harmonic mean of the grid line intersecting the capillary segments. We estimated the length of the glomerular capillaries in individual capillary tufts imaged by SEM on the basis of the resin volume (volume of the capillary lumen) and the mean capillary segment area calculated from mean capillary diameter. We calculated the length of single glomerular capillary as the ratio between lumen volume and mean capillary lumen area. To estimate the effective volume occupied by the resin (corresponding to capillary lumen volume) within the capillary tuft we obtained images of cross sections of the glomerular casts using a focused ion beam (FIB) cut performed on a SEM-FIB electron microscope (Cross-Beam 1540EsB, Carl Zeiss GmbH, Oberkochen, Germany). Digitized images were then processed for morphometric analysis to obtain, by point counting (superimposing a 20 x 29 orthogonal grid), the ratio between resin volume and total capillary tuft volume, assuming volume density equal to area density.

PCR Array. For real-time PCR array, total RNA was extracted from rat kidney specimens using Qiagen RNeasy Mini kit. RNA extracted from three different animals for each group was pooled and 1 μg used for the first-strand cDNA synthesis using the RT^2 First Strand Kit (Invitrogen). The Rat Angiogenesis Pathway RT^2 Profiler PCR Array (PARN-024Z; SABiosciences, Qiagen) was used to determine the differentially expressed genes. Data analysis was performed using the online SA bioscience software. The boundary (fold regulation cut off) was set to 2-fold change between the different groups.

Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from kidney of each rat MWF or Wistar using Trizol reagent and contaminating genomic DNA was removed by RNase-free DNase (Promega) for 1 hour at 37°C. The first-strand cDNA (2 µg) was produced using SuperScript VILO cDNA Synthesis Kit (Life Technologies) following the manufacturer's procedure. No enzyme was added for reverse transcriptase-negative controls (RT-). Amplification was performed on ViiA7 Real Time PCR System with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s to reach the plateau. The comparative Ct method normalizes the number of target gene copies to the housekeeping gene as Gapdh (ΔCt). Gene expression was then evaluated by the quantification of cDNA corresponding with the target gene relative to a calibrator sample (Wistar rats) serving as physiologic reference (ΔΔCt). On the basis of exponential amplification of target gene as well as calibrator, the amount of amplified molecules at the threshold cycle is given by: 2^-ΔΔCt. The following primers (300 nM) were used: rat TGF-β2 sense 5'-TAAAATCGACATGCCGTCCCA-3', antisense 5'-GGATGGCATCAAGGTACCCAC-3'; rat ET-1 sense 5'- CTGTTTGTGGCTTTCCAAGGA-3', antisense 5'-CTCTGCTCCCAAGACAGCTGT -3'; GAPDH sense 5'-TCATCCCTGCATCCACTGGT-3', antisense 5'- CTGGGATGACCTTGCCCAC-3'.

Renal histology. Kidney specimens were fixed in Duboscq-Brazil and embedded in paraffin. Three-micron sections were stained with periodic acid-Schiff (PAS) reagent and the incidence and extent of glomerular and tubular structural lesions were estimated at light microscopy (ApoTome Axio Imager Z2, Zeiss, Jena, Germany). An average of 35 glomeruli was examined for each animal. Each glomerulus was scored according to the extent of sclerotic changes consisting of matrix deposition, capillary

occlusion and capillary tuft adhesion to Bowman's capsule. Score was assigned to 0 in the absence of sclerosis, to 1 for changes affecting less than 25% of the glomerular area, to 2 and 3 for lesions affecting 25–50 and 50–75% of the tuft, respectively, and to 4 for lesion exceeding 75% of the tuft. The average GS index in each animal was then calculated as weighted mean. Tubular structural changes were evaluated by semiquantitative scores. Tubular atrophy, interstitial fibrosis and inflammation and luminal casts were graded from 0 to 3+ (0=no changes; 1+=changes affecting less than 25% of the sample; 2+=changes affecting 25 to 50% of the sample; 3+=changes affecting more than 50% of the sample). At least 10 fields per kidney slice were examined for histological scores at low-power magnification. All tissue sections were analyzed by the same pathologist in a single-blinded manner.

Immunofluorescence experiments. PLP or Acetone-fixed cryosections were post-fixed with cold acetone, treated with 1% bovine serum albumin (BSA) to block non-specific sites and incubated with the following antibodies: mouse anti-Reca1 (1:50, AbDSerotec, Kidlingdon, Oxford, United Kingdom), Cy3-conjugated mouse anti-αSMA (1:100, Sigma Aldrich, St Louis, MO, USA), rabbit anti-ki67 (1:200, AbCam, Cambridge, UK), rabbit anti-caspase3 (1:100, Cell Signaling, Beverly, MA, USA), rabbit anti-ET_AR (1:200, Alomone Labs, Jerusalem, Israel), goat anti-ET1 (1:100, Santa Cruz), rabbit anti-α-actinin4 (1:150, OriGene Technologies, Rockville, MD), followed by the specific FITC, Cy3 or Cy5-conjugated secondary antibodies. Nuclei were stained with DAPI, and renal structures with FITC wheat germ agglutinin (WGA). Negative controls were obtained by omitting primary antibodies on adjacent sections. Fluorescence was examined by confocal laser microscopy (LS 510Meta, Zeiss). Capillary volume density (Vv) was quantified as Reca1–positive vessels in 20 tubulointerstitial fields and 20 glomeruli per sample (n=4 for each group) randomly

acquired. By using the analysis software ImageJ 1.40g, digitized images were binarized using a threshold for areas of Reca1 staining, and the values were expressed as percentage of area occupied by Reca1 on total area of the acquired field. Reca1-αSMA co-staining was evaluated in an average of 10 glomeruli and 10 high power fields of interstitial area (HPF, x40) in each kidney sample. Merge signal was quantified using an appropriate software (Axio Vision, Apotome Axio Imager Z2, Zeiss) and expressed as percentage of Reca1/αSMA-positive area on total Reca1-positive area *per* field. Interstitial fibrotic vessels were identified as large vessels with an abnormal αSMA accumulation, and quantification was expressed as percentage on the total number of Reca1+ large vessels. Endothelial cell proliferation was quantified as the number of Reca1+Ki67+ cells in each field (40X). Reca1-caspase3 and Reca1-ET_AR expression were evaluated as semi-quantitative score of the merge positive area per HPF (0: no signal, 1: occasional, 2: mild, 3: diffuse).

Immunoperoxidase experiment. Formalin fixed, paraffin embedded kidney sections (3 µm) were deparaffinized, hydrated and incubated for 30 minutes with 0.3 % H_2O_2 in methanol to quench endogenous peroxidase. Antigen retrieval was performed by boiling sections using microwave (twice for 5 min in citrate buffer 10 mM, pH 6.0 at operating frequency of 2450 MHz and 600 W power output). Sections were blocked with 1% bovine serum albumin (BSA), and subsequently incubated overnight with rabbit anti-NRF2 antibody (diluted 1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation with specie-specific biotinylated secondary antibody and diaminobenzidine (DAB, Merck, Darmstadt, Germany) substrate solution, slides were counterstained with hematoxylin, dehydrated in graded alcohols, mounted with coverslips and observed by light microscopy (Primo Star, Zeiss, Jena, Germany). Negative controls were obtained by omitting the primary antibody on

adjacent sections. Nrf2 signal was graded on a scale from 0 to 3 on the basis of the number of positive structures in each field (0: no signal, 1: from 1 to 5, 2: from 6 to 10, 3: more than 10).

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