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Original Paper

Effects of Perfusion Pressures on Podocyte Loss in the Isolated Perfused Mouse Kidney

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Key Words
Glomerular hypertension • Glomerular hyperfiltration • Mechanical stress • Progression • CKD • Chronic kidney disease

Abstract
Background/Aims: Podocytes are lost in most glomerular diseases, leading to glomerulosclerosis and progressive kidney disease. It is generally assumed, that podocytes are exposed to the filtration flow and thus to significant shear forces driving their detachment from the glomerular basement membrane (GBM). In this context, foot process effacement has been proposed as potential adaptive response to increase adhesion of podocytes to the GBM. ***Methods:*** We have tested these hypotheses using optical clearing and high-resolution 3-dimensional morphometric analysis in the isolated perfused murine kidney. We investigated the dynamics of podocyte detachment at different perfusion pressures (50, 300 and more than 450 mmHg) in healthy young or old mice (20 vs. 71 weeks of age), or mice injected with anti-GBM serum to induce global foot process effacement. ***Results:*** Results show that healthy podocytes in young mice are tightly attached onto the GBM and even supramaximal pressures did not cause significant detachment. Compared to young mice, in aged mice and mice with anti-GBM nephritis and foot process effacement, gradual progressive loss of podocytes had occurred already before perfusion. High perfusion pressures resulted in a relatively minor additional loss of podocytes in aged mice. In mice with anti-GBM nephritis significant additional podocyte loss occurred at this early time point when increasing perfusion pressures to 300 mmHg or higher. ***Conclusion:*** This work provides the first experimental evidence that podocytes are extraordinarily resistant to acutely increased perfusion pressures in an *ex vivo* isolated kidney perfusion model. Only in glomerular disease, significant numbers of injured podocytes detached following acute increases in perfusion pressure.

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Introduction

Glomerular hypertension and hyperfiltration damage the glomerulus leading to progressive glomerular disease. It is widely believed that increased perfusion pressure exposes the tuft to increased mechanical stress challenging the adhesion of podocytes to the glomerular basement membrane (GBM), contributing to their detachment [1, 2].

Podocytes are highly differentiated, post-mitotic cells, essential for an intact glomerular filtration barrier. Podocyte loss is most likely the result of detachment of viable cells from the GBM rather than apoptosis or necrosis [3-6] as it has been possible to culture podocytes recovered from the urine of patients [4]. Detachment of viable podocytes from the GBM may result from impaired attachment mechanisms or altered mechanical forces. Impaired attachment to the GBM may be due to cellular injury or reduced expression of adhesion molecules and is mainly associated with inflammatory glomerular diseases. With respect to mechanical forces relevant for the podocyte, there are two key determinants, namely filtration pressure and filtrate flow [7-9]. Filtration pressure generates circumferential wall stress and acts as a distending force on the GBM. Variations in transmural hydrostatic pressure are compensated effectively by the ability of the GBM to act as an elastic membrane and expand or to shrink in surface area [8, 10]. On the other hand, filtrate flow across the GBM exerts tangential forces on the surface of podocytes, i.e. shear stress. We know from *in vitro* studies that podocytes are highly susceptible to shear stress detaching from their substrate when exposed to shear stresses more than 0.025 Pa [11] and adapting an intermediate phenotype that may help them to counteract flow-derived forces [12]. Whether glomerular hypertension affects podocytes via an increased filtration pressure and/ or increased filtration remains still unclear.

In this study, we provide the first analysis of the capability of podocytes to resist increased mechanical forces in an *ex vivo* model. Podocyte loss was quantified with high resolution using tissue clearing and 3-dimensional morphometric analysis in young and healthy mice, in aged mice and mice with diffuse podocyte effacement induced by an anti-GBM serum.

Materials and Methods

Animals

All animal experiments were conducted according to the guidelines of the German law for the welfare of animals, and were approved by *Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen* (Az 84-02.04. 2015.A469). Mice were housed in a specific pathogen-free facility with free access to chow and water and a 12-hour day/night cycle. Breeding and genotyping were done according to standard procedures. Pod-rtTA/LC1/R26R/H2BeGFP mice on a FVB/N genetic background have been described before [13]. To activate transgene expression of Pod-rtTA-eGFP, mice received doxycycline via the drinking water ad libitum for 7 days (2 g/l, 5% sucrose, protected from light) followed by a washout period of 1 week as per previous reports. Anti-GBM nephritis was induced by a single intraperitoneal injection of 5 mg/g bodyweight nephrotoxic serum as previously described [14].

Kidney perfusion

The isolated kidney perfusion model was used as described elsewhere [15]. Briefly, the mice were narcotized with an intraperitoneal injection of xylazin/ketamine (0,1ml/10g body weight i.p.), and then both kidneys were perfused with a complex modified Krebs-Henseleit-solution (Table 1) supplemented with 5% bovine serum albumin (BSA) at 37°C to mimic the physiological milieu during the experiment. Maximal vasodilatation of the kidney vasculature was induced by 1. subcutaneous injection of verapamil (50µl) immediately after induction of anesthesia and 2. addition of papaverine to the perfusion solution. After initial perfusion for 5 minutes at 50 mmHg a fluorescently-labeled lectin (Communis I - RCA120; Vector Laboratories: RL-1082; 4 µl in 986 µl of saline) was injected to label endothelial cells. Kidneys were perfused for additional 5 minutes with the modified Krebs-Henseleit-solution with 5% BSA. After that, the left kidney which served as paired control in every experiment was removed after careful ligation of the left

renal vessels, cut into slices, and directly immersed in 3% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The right kidney was perfused either for additional 5 minutes at 300 mmHg or with supramaximal pressure (>>300mmHg, filtration pressures were estimated to be well above 400 mmHg), applied manually, with a total volume of 50 ml perfusion solution. The kidneys were perfused with a constant pressure of 50 mmHg or 300 mmHg by using a pressure-controlled pump (Universal Perfusion Systems UP-100, Hugo-Sachs Electronics, Germany). Then the right kidney was removed, cut into 2 mm slices and immersed in 3% paraformaldehyde (PFA) in phosphate-buffered saline (PBS).

Table 1. Modified Krebs-Henseleit-Solution. EDTA, Ethylenediaminetetraacetic acid

Components	g/l
NaCl	5,600
KCl	0,150
KH_2PO_4	0,200
$MgSO_4$	0,190
$NAHCO_3$	2,600
Glucose	1,300
$CaCl_2$	0,280
EDTA	0,001
Creatinin	0,015
Urea	0,170
Pyruvate	0,078
Glutamate	0,300
Malate	0,112
Papaverin	0,037
Ketoglutarate	0,220
Aminoplasmal 10% (ml)	8
Albumin	50
Insulin IE	4
Ampicillin	0,030
Flucloxacillin	0,030
Verapamil (ml)	0,200
Furosemide	0,200
Heparin 5000 IE	1
Distilled water	991
pH: 7.4	

3D imaging and computational analysis

PFA-immersed kidney slices were incubated on a mechanical shaker for 5 days at room temperature. Thereafter, fixed samples were washed in 1x PBS and placed in a mechanical shaker overnight at room temperature. Kidney slices were then placed in high-grade 100% ethanol (Merck; 100983) for 1 hour at room temperature with gentle shaking (at least 1 change to fresh ethanol), followed by direct immersion in Ethyl Cinnamate (ECi) (Sigma-Aldrich, St. Louis, MO; 112372) and were left overnight in a gentle shaker at room temperature under light protection. Tissue translucency was achieved in less than 1 hour. For upright microscopes, we used disposable in-house-built chambers as previously described [16]. Most experiments were performed using a 2-photon microscope (*LaVision BioTec TriMScope*, "Medizinische Fakultät RWTH Aachen, IZKF Aachen, Core Facilities). Fiji imaging software was used to move through the Z-axis of each stack of serial optical sections and to isolate individual glomeruli via 3D cropping [16]. 40 glomeruli per kidney (20 subcortical and 20 juxtamedullary) were selected for analysis. Thus, in total 1200 glomeruli were analyzed in this study to obtain results with sufficient precision. Podocytes were identified as $eGFP^+$ cells. Quantification of capillary volume, glomerular volume as well as nuclei number (dots) and volume was performed using a 3D rendering and analysis software (Imaris v9.1; Bitplane AG, Zurich, Switzerland). The distance of podocytes from the vascular pole was calculated using Bitplane XTension "Spot Closest. Distance". Automated quantification of podocytes was performed as previously described [17]. Image analysis was performed using Imaris (Biplane AG Zurich, Switzerland). Each glomerulus was defined by its lectin-labeled afferent arteriole. 20 subcortical and 20 juxtamedullary (defined by their distance from the cortical surface).

Light microscopy & Immunofluorescence

For light microscopy, the 4% buffered formalin-fixed kidney fragments were dehydrated, embedded in paraffin and stained with periodic acid–Schiff (PAS). The percentage of abnormal/injured glomeruli was calculated based on the identification of 50 representative glomerular cross-sections per mouse selected during a systematic walk across the renal cortex. Our standard immunofluorescence protocol [18] was performed on 2 µm paraffin-embedded sections. The following antibodies were used: chicken polyclonal anti-GFP (ab13970; Abcam), mouse monoclonal anti-synaptopodin (sc-515842; Santa Cruz Biotechnology), polyclonal rabbit anti-mouse p57 (sc-8298; Santa-Cruz Biotechnology), rabbit polyclonal anti-WT1 (sc-192; Santa-Cruz Biotechnology), Cy2 donkey anti-chicken (703-225-155; Dianova, Hamburg, Germany), Alexa Fluor546 goat anti-mouse IgG1(A-21123; ThermoFischer), polyclonal donkey anti-rabbit AF555 (A31572; Life Technologies, Carlsbad, CA).

Electron Microscopy

Small pieces of the cortex were fixed in Karnovsky-solution and embedded in Epon (Serva, Heidelberg, Germany). Ultrathin sections were examined with a transmission electron microscope ZEISS Leo 906 at 60 kV by a magnification from 3597-6000x. Samples were washed in 0.1 M Soerensen's phosphate buffer (Merck, Darmstadt, Germany), post-fixed in 1% OsO_4 (Roth, Karlsruhe, Germany) in 17% sucrose buffer

(Merck, Darmstadt, Germany) and dehydrated by ascending ethanol series (30, 50, 70, 90 and 100%) for 10 min each. The last step was repeated 3 times. Dehydrated specimens were incubated in propylene oxide (Serva, Heidelberg, Germany) for 30 min, in a mixture of Epon resin (Serva, Heidelberg, Germany) and propylene oxide (1:1) for 1h and finally in pure Epon for 1h. Epon polymerization was performed at 90°C for 2h. Ultrathin sections (70-100 nm) were cut by ultramicrotome (Reichert Ultracut S, Leica, Wetzlar, Germany) with a diamond knife (Leica) and picked up on Cu/Rh grids (HR23 Maxtaform, Plano, Wetzlar, Germany). Contrast was enhanced by staining with 0.5% uranyl acetate and 1% lead citrate (both EMS, Munich, Germany). Samples were viewed at an acceleration voltage of 60 kV using a Zeiss Leo 906 (Carl Zeiss, Oberkochen, Germany) transmission electron microscope.

Statistical analysis

Statistical analyses were performed with GraphPad Prism v8 software. All values are expressed as means ± SD. For a comparison of 2 groups, a *t*-test was used. Comparison of several groups was performed using analysis of variance; *post hoc* Tukey correction was used for multiple comparisons. All tests were 2-tailed and statistical significance was defined as $P < 0.05$.

Results

Semi-automatic method to determine the number of podocytes per glomerulus

Three groups were studied, i.e. healthy young and old mice and mice with anti-GBM glomerulonephritis (♂:♀ 1:1). Young, healthy mice were 15-21 weeks old, whereas old (aged) mice were 74 weeks old. Global foot process effacement was induced in 14-20 week old mice via a single injection of anti-GBM serum 3 days before setting up the isolated perfused kidney model (Fig. 1A), as previously described [19].

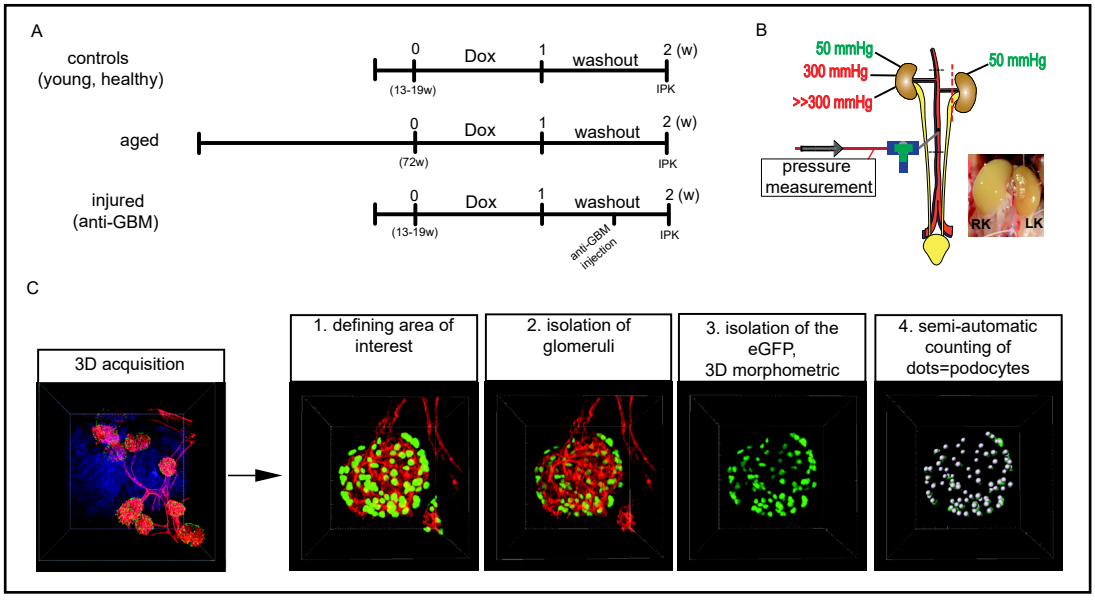

Fig. 1. Experimental setup. (A) Experimental design using the indicated mouse models. (B) Schematic of the perfusion circuit and the ligatures placed during surgery. Perfusion buffer was pumped at controlled perfusion pressures, as verified by a pressure transducer. Both kidneys were perfused for 5 minutes at 50 mmHg. Next, the left kidney was removed and the right kidney was perfused either for additional 5 minutes at 300 mmHg or with supramaximal pressure (>>300 mmHg). The black lines show the area of the respective ligature. Inset: View through the microscope into the murine situs after perfusion with 50 mmHg (left kidney, LK) and >>300 mmHg pressure perfusion of the right kidney, which is enlarged due to edematous swelling. (C) Semi-automatic method to determine the number of podocytes per glomerulus involves 3D-acquisition and computational analysis; DOX: doxycycline; green valve: perfusion cannulation.

To determine the influence of intrarenal perfusion pressure on podocytes, kidneys were subjected to different perfusion pressures in the isolated kidney perfusion model. First, both kidneys were always perfused at 50 mmHg for 5 min. To test whether our perfusion solution preserved the integrity of the glomerulus in the viable unfixed kidneys, 2 mice were perfused at a constant pressure of 100 mmHg for 90 min. Perfusion, urinary flow (25 µl/min/gr body weight) as well as inulin clearance (22 µl/min) remained stable over the entire time (Supplementary Fig. 1 – for all supplementary material see www.cellphysiolbiochem.com). In experimental mice, after initial perfusion at 50 mmHg, the left kidney was removed and served as a control in all experiments. Next, a constant pressure of 300 mmHg or a pressure significantly higher than 300 mmHg (>>300 mmHg; supramaximal filtration pressure of about 450 – 500 mmHg) were applied to the remaining right kidney for 5 min. (Fig. 1B).

In the isolated perfused kidneys, podocytes were identified using nuclear labeling with eGFP-histone in our transgenic mice expressing eGFP under the podocin promoter (eGFP:Pod-rtTA mice). Podocyte numbers were quantified using a combination of this *in vivo* metabolic labeling and 3D morphometry (Fig. 1C).

Podocyte loss in healthy and aged mice

Healthy mice contained 87.46 ± 5.66 (SD) podocytes per glomerulus at baseline (i.e. at physiological perfusion pressure of 50 mmHg) (Fig. 2A). Juxtamedullary glomeruli were larger than those in the outer cortex (Supplementary Fig. 2), but this was not associated with higher podocyte numbers (87.0± 23.65 vs. 90.1 ± 18.49, respectively, Fib 2B). No differences in the number of podocytes related to sex were observed (see below).

In young mice, there was no significant loss of podocytes at high (300 mmHg) or maximal (>>300 mmHg) perfusion pressures (85.62 ± 10.83 and 81.98 ± 6.45 podocytes per glomerulus, respectively) (Fig. 1A). On PAS sections, mild histological changes were observed particularly in aged mice (Fig. 2A), which were independent of perfusion pressures. In addition, tubular dilatation and interstitial edema were apparent as a result of perfusion.

In humans, older age (53 ± 10 years) has been associated with absolute and relative podocyte depletion [20]. Compared to young mice, our mice aged 72 weeks contained significantly lower numbers of podocytes per glomerulus at baseline (52.81 ± 13.8 SD), indicating that about 40% of the podocytes had been lost. Perfusion at high or maximal pressures resulted in only a very mild reduction of podocyte numbers (48.65 ± 16.8 SD and 41.06 ± 17.7 SD, respectively). Because of the precision of the counting method (absolute podocyte numbers in 40 glomeruli per mouse) [21], the loss of podocytes of 7.9% and 22% after perfusion with 300 or >>300 mmHg respectively compared to baseline suggests that this minor increase in podocyte loss with higher pressure is not a coincidental finding. On PAS sections, mild glomerular changes were observed in aged mice at baseline (mesangial expansion and occasional sclerosis, Fig. 2F). After perfusion tubular dilatation and some interstitial edema were observed (Fig. 2G-H).

Podocyte loss after acute injury

Anti-GBM serum was applied to young healthy mice to induce significant and specific injury to podocytes. At the very early time-point (i.e. 3 days) after induction of anti-GBM glomerulonephritis, generalized foot process effacement was observed in all glomeruli by transmission electron microscopy (Fig. 3A-D). In isolated perfused kidneys obtained from these mice, at baseline (i.e. perfusion with 50mmHg) podocyte numbers per glomerulus were reduced by 20% compared to controls (69.76 ± 7.07 vs 87.46 ± 5.66, respectively) (Fig. 3E). Perfusion with higher pressures (300 and >>300 mmHg) resulted in significant further reductions of podocyte numbers per glomerulus (50.84 ± 7.82 and 53.18 ± 4.26, respectively), i.e. 27% and 24% additional reduction (Fig. 3E). When analyzing individual glomeruli, the anti-GBM mice, perfusion with >>300 mmHg caused more than 80% podocyte loss in a minority of the glomeruli (Fig. 3E). This is in line with the focal nature of the anti-GBM disease model. Within juxtamedullary glomeruli, podocyte loss was more pronounced compared to subcortical glomeruli (56.66 ± 16.9 and 47.35 ± 17.07, respectively) (Fig. 3F).

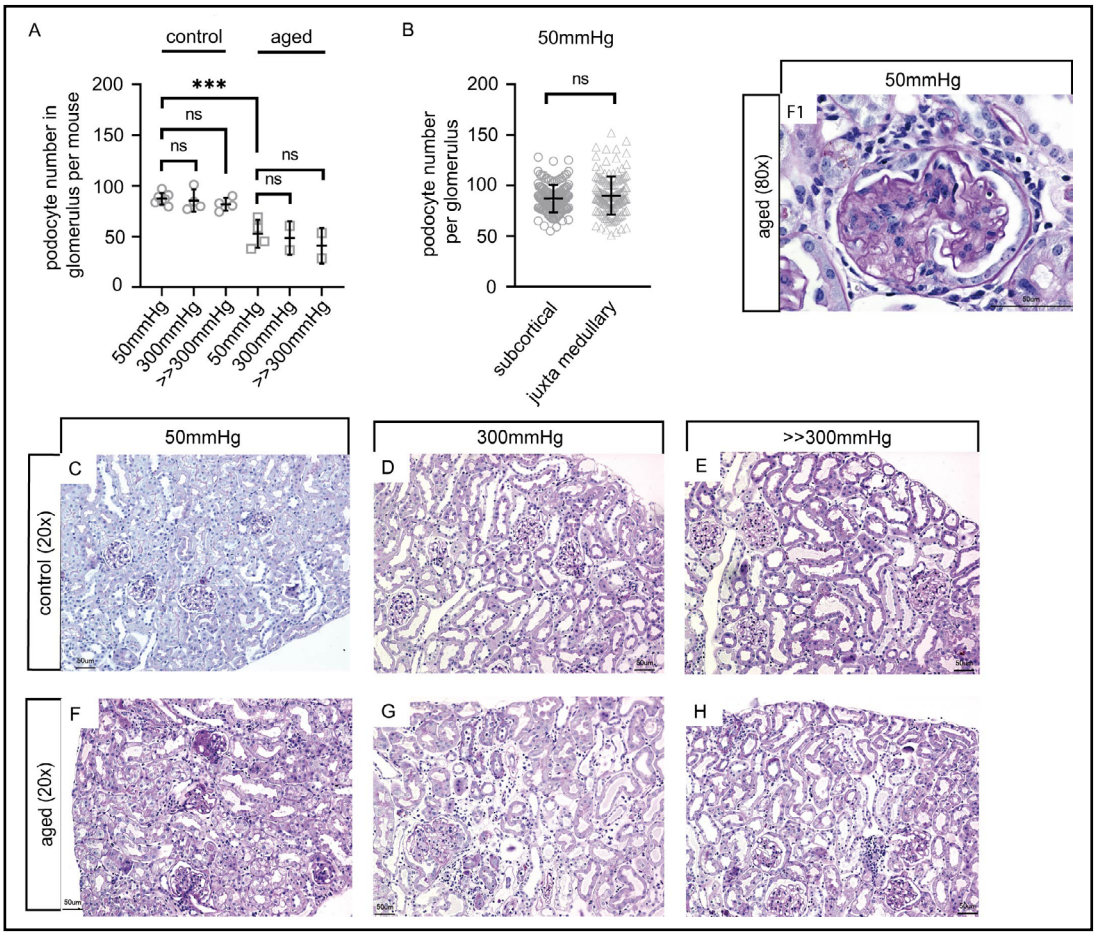

Fig. 2. Podocyte loss in healthy mice. (A) Total podocyte number per mouse at baseline and after high (300 mmHg) or maximal perfusion pressures (>>300 mmHg) (each circle represents 1 kidney (40 glomeruli pro mouse, n=7 control mice, n=4 aged mice), ANOVA, ***P<0.001 and ns = not statistically significant; error bars represent means ± SD). (B) Total podocyte number per glomerulus in subcortical and juxtamedullary glomeruli at baseline (each circle represents 1 subcortical glomerulus and each triangle 1 juxtamedullary glomerulus (n=20 per mouse), ANOVA, ns = not statistically significant; error bars represent means ± SD). (C-E) Histologic staining of young mice with periodic acid-Schiff at baseline and after perfusion with higher pressures (300mmHg and >>300mmHg), showing only mild changes, i.e tubular dilatation and tubulointerstitial edema. (F-H) Histologic staining of aged mice at baseline showing mesangial expansion and occasional sclerosis (arrow, F1) and after perfusion with 300mmHg and supramaximal pressure showing tubulointerstitial dilatation (G-H); ctrl: control mice.

By transmission electron microscopy (TEM), specific changes were observed after high perfusion pressures, in particular focal podocyte detachment from the GBM and denuded basement membrane (Fig. 3D). Cellular debris was present within the capillaries as well as within Bowman's space (>>300 mmHg); the endothelium did not show significant changes. PAS staining identified tubular dilation. Three days after the injection of anti-GBM serum, no crescentic lesions had developed yet (Fig. 3G-I).

Males showed a higher degree of podocyte loss compared to females (63.71 ± 2.11 vs 75.81 ± 3.83 podocytes per glomerulus), which is consistent with the observation that male mice are more susceptible to anti-GBM antiserum and form more cellular crescents in the course of the disease (Fig. 3J). However, the additional loss of podocytes after high perfusion pressures was similar in males and females (Fig. 3J).

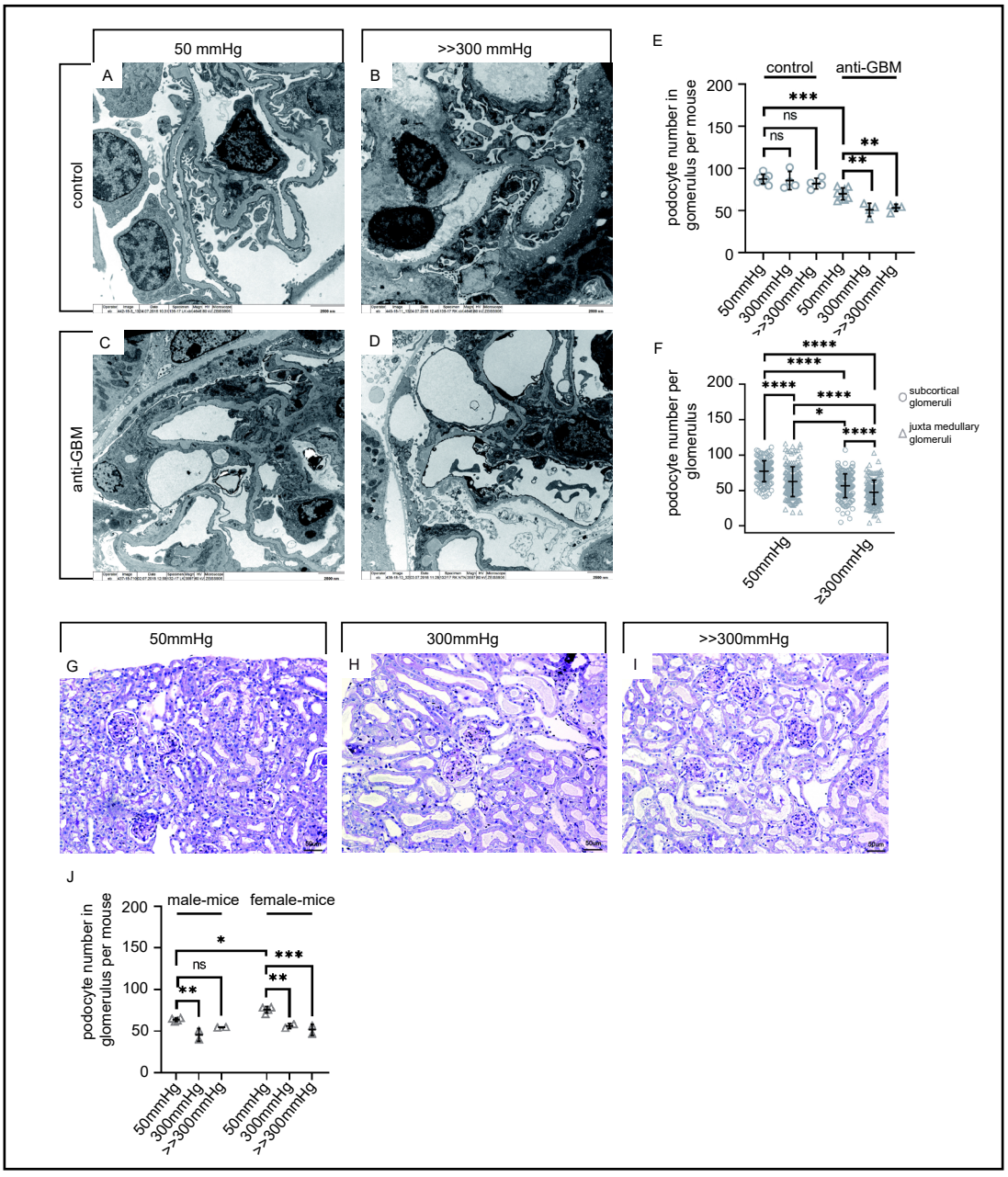

Fig. 3. Podocyte loss after acute injury. (A-B) Transmission electron microscopy from a control kidney perfused at a low pressure and after supramaximal pressure (>>300 mmHg) showing normal foot process architecture. (C-D) Transmission electron microscopy reveals the typical finding of foot process effacement after injection of anti-GBM serum at baseline. After perfusion with higher pressure, areas of denuded basement membrane were observed. (E) Total number of podocytes per mouse in anti-GBM mice at baseline and under higher (300 mmHg) or supramaximal pressures (>> 300 mmHg); (each circle represents 1 kidney; n=7 control kidneys with 50mmHg, n=8 anti-GBM kidneys with 50mmHg, n=4 kidneys with 300 mmHg or >> 300mmHg). (F) Total number of podocytes per glomerulus in juxtamedullary and subcortical glomeruli; each circle represents 1 subcortical glomerulus (n= 20 glomeruli pro mouse) and each triangle represents 1 juxtamedullary glomerulus (n= 20 glomeruli pro mouse). (G-I) Histologic staining of anti-GBM mice at baseline identified protein casts, whereas three days after injection of anti-GBM serum no crescentic lesions could be found. After perfusion with higher pressure tubule-interstitial dilatation and edema were observed. (J) Total podocyte number per mouse between males and females; each circle represents 1 kidney (n=4 control kidneys with 50mmHg, n=4 anti-GBM kidneys with 50mmHg, n=2 kidneys with 300 mmHg or >> 300mmHg). For multiple comparisons ANOVA. ****P<0.0001, ***P < 0,0001, **P<0.01, *P<0.05 and ns and ns = not statistically significant; error bars represent means ± SD.

Tracing the lost podocytes in the tubules

eGFP$^+$ podocytes were regularly observed within the lumen of the proximal tubules where they appeared to adhere to the brush-border of proximal tubule cells (Fig. 4A). These podocytes were not washed away in hyperperfused kidneys, instead they adhered rather tightly to the brush border to withstand the extraordinary flow of the primary urine at very high perfusion pressures. To confirm their identity, podocytes were co-stained with podocyte-specific markers (i.e., p57, synaptopodin and WT-1) in addition to the genetic lineage tracing marker nuclear eGFP (Fig. 4B). Podocyte numbers were significantly lower in those glomeruli with adhering podocytes in the associated proximal tubule, and the presence of podocytes in tubules inversely correlated with the number of podocytes per glomerulus (Fig. 4C).

Preferential loss of podocytes in perihilar location

Finally, we investigated whether podocyte loss driven by an acute increase in perfusion pressures might occur in preferential locations of the glomerulus (i.e. at the vascular pole vs. the tubular pole of the glomerular tuft). To analyze the spatial distribution of podocyte detachment, the vascular pole was marked in each glomerulus (acquired in 3D) based on the lectin signal and the distance of each podocyte from the vascular pole was determined semi-automatically. The individual distances from the vascular pole were separated into quartiles (Fig. 4E). As depicted in Fig. 4E, the majority of podocytes clustered in the 2 middle quartiles (i.e. quartile 2 and 3). Less than 12 % of the podocytes localized to either in the first, i.e. vascular pole, or the fourth quartile, i.e. most distant from the vascular pole. Upon perfusion with pressures >> 300 mmHg, no significant changes in the spacial distribution of podocytes were observed in young and healthy mice. In aged mice, podocyte distances from the vascular pole were generally increased, most likely reflecting glomerular hypertrophy, whereas hyperperfusion resulted in a preferential detachment of podocytes close to the vascular pole (1st quartile reduced from 11.5 to 4.9% in aged mice, Fig. 4E). Similarly, a preferential detachment of podocytes close to the vascular pole was observed in effaced podocytes (anti-GBM, 1st quartile reduced from 25 to 5%).

Discussion

In this study, we examined the acute effects of elevated filtration pressure and filtration flow on podocyte loss in the isolated perfused murine kidney, using the currently most accurate method to determine podocyte numbers.

The first major finding of our study was that healthy podocytes are not susceptible to acute shear stress *in vivo*, as suggested previously by cell culture studies [11]. To our surprise, not even extreme supraphysiological pressures (more than 450 mmHg) induced significant podocyte detachment. Rather, transmission electron microscopy revealed that all three layers of the filtration barrier remained relatively well preserved. Older mice showed reduced podocyte numbers (as reported previously) and this was associated with only very limited increased susceptibility to high perfusion pressures.

The second major finding was that prior podocyte injury rendered podocytes susceptible to detachment at high perfusion pressures. Anti-GBM mice showed global foot processes effacement three days after injection of the anti-GBM antiserum and significant numbers detached at high perfusion pressures. In aged mice, we hypothesize that podocyte physiology is relatively well preserved, rendering them less susceptible to detachment.

By applying a semi-automated counting approach, each glomerulus was analyzed separately (total of 1200 glomeruli analyzed). We confirmed that juxtamedullary glomeruli have a higher volume than subcortical glomeruli. Interestingly, podocyte numbers were the same, showing that podocyte density is reduced in juxtamedullary glomeruli. In addition, juxtamedullary glomeruli were also more vulnerable to increased filtration pressures compared to subcortical glomeruli. These findings could explain in part, why FSGS lesion can be observed at a higher frequency in such larger glomeruli [22, 23].

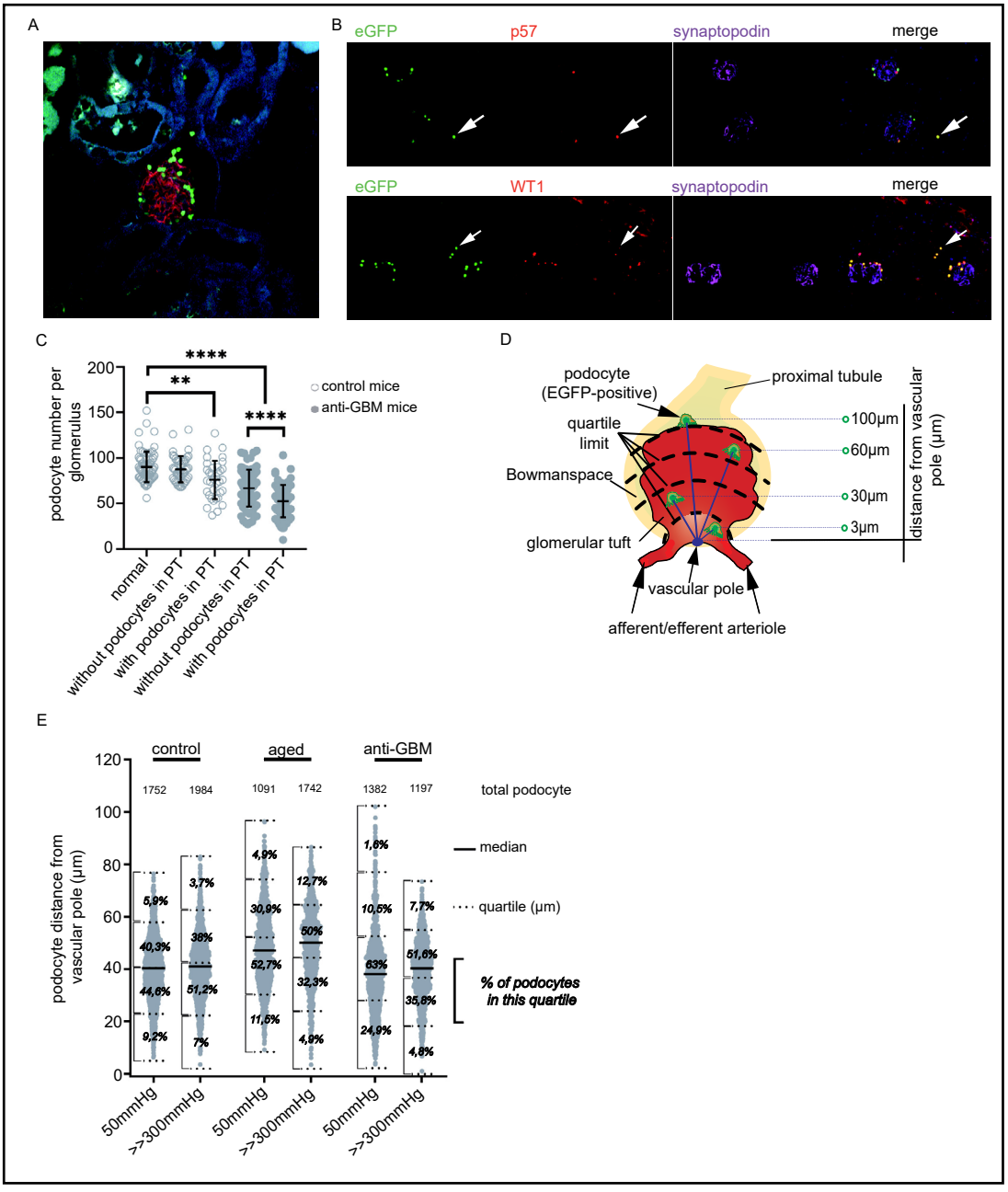

Fig. 4. Detection of podocytes in the adjacent prox. tubule. (A) Representative image showing eGFP+ podocytes (green) in the tubulus. (B) Immunofluorescence staining of podocytes co-labeled by the transgene histone-eGFP (green), and the endogenous podocyte markers p57 (red), wt-1 (red) and synaptopodin (purple). (C) Association of the number of podocytes per glomerulus with the number of podocytes identified in the tubule (n=81 glomeruli from control mice, n= 41 glomeruli form aged mice and n= 81 glomeruli from anti-GBM mice). For multiple comparisons ANOVA was used. ****P<0.0001, **P< 0.01 and ns = not statistically significant; error bars represent means ± SD; in the graph each circle represents 1 podocyte; (D) schematic showing how distance of podocytes from vascular pole was calculated. (E) Distances of individual podocytes from the vascular pole. The individual distances from the vascular pole were separated into quartiles.

A limitation of this study is the short observation period, as no conclusions can be made regarding the long-term adaptation of podocytes to increased filtration pressures. It has been proposed that the first response of podocytes exposed to shear stress is to undergo *protective* structural changes [8, 24]. These changes include the loss of filtration slits (i.e. FPE) and replacement of slit-diaphragms by occluding or tight junctions. Occluding junctions have been previously described in several glomerular disease models [25-28]. However, in our study we observed that podocytes with FPE were more susceptible to higher perfusion pressures. Hence, our results showed that these adaptive changes were not enough to protect against detachment or potentially even the opposite, that they render podocytes more susceptible to detachment.

Next, it may be argued that the isolated perfused kidney is not a physiological system and that the pressures applied were much higher than under *in vivo conditions*. To our knowledge, no other model using an intact viable kidney exists that would allow investigating the effects of increased perfusion pressures directly. By application of vasodilators before and during perfusion, maximal dilatation of pre-glomerular vessels as induced to allow unrestricted flow (hyperfiltration) and maximal transmission of increased perfusion pressures into the glomerulus.

In the present study, the acute effects of higher perfusion pressures were studied. Long term effects of pathological hyperperfusion could be more deleterious, leading to even more podocyte loss. However, our study suggests, that this chronic loss of podocytes is not driven by the shear force of filtration flow per se but much rather by the (mal-)adaptive changes in podocytes which decrease their adherence to the GBM. Patients with glomerular diseases benefit from antihypertensive treatment by preventing (mal-)adaptive structural changes of podocytes.

Conclusion

This work provides the first *in vivo* evidence that healthy podocytes are extraordinarily firmly attached to the GBM in healthy mouse kidneys and can resist even very high perfusion pressures. Aging or even more pronounced acute podocyte injury with global effacement, renders podocytes susceptible to detachment at increased perfusion pressures.

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Author Contributions

TS performed the experiments and analyzed the data. All authors revised the manuscript before submission. VGP, VS, KL, TS, MB, ES and MJM assisted with experiments and data analyses. VGP, JF, PB, RH, MV, ES and MJM provided experimental resources and expertise. ES and MJM were responsible for the experimental design, writing of the manuscript, and study supervision.

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Statement of Ethics

Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body.

Disclosure Statement

The authors declare that they have no conflicting interests.

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