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Plasminogenuria is associated with podocyte injury, edema and kidney dysfunction in incident glomerular disease

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Abstract

Urinary plasminogen/plasmin, or plasmin(ogen)uria, has been demonstrated in proteinuric patients and exposure of cultured podocytes to plasminogen results in injury via oxidative stress pathways. A causative role for plasmin(ogen) as a “second hit” in kidney disease progression has yet to have been demonstrated *in vivo*. Additionally, association between plasmin(ogen)uria and kidney function in glomerular diseases remains unclear. We performed comparative studies in a puromycin aminonucleoside (PAN) nephropathy rat model treated with amiloride, an inhibitor of plasminogen activation, and measured changes in plasmin(ogen)uria. In a glomerular disease biorepository cohort (n=128), we measured time-of-biopsy albuminuria, proteinuria, and plasmin(ogen)uria for correlations with kidney outcomes. In cultured human podocytes, plasminogen treatment was associated with decreased focal adhesion marker expression with rescue by amiloride. Increased glomerular plasmin(ogen) was found in PAN rats and focal

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AUTHOR CONTRIBUTIONS

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DISCLOSURES

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segmental glomerulosclerosis (FSGS) patients. PAN nephropathy was associated with increases in plasmin(ogen)uria and proteinuria. Amiloride was protective against PAN-induced glomerular injury, reducing CD36 scavenger receptor expression and oxidative stress. In patients, we found associations between plasmin(ogen)uria and edema status as well as eGFR. Our study demonstrates a role for plasmin(ogen)-induced podocyte injury in the PAN nephropathy model, with amiloride having podocyte-protective properties. In one of the largest glomerular disease cohorts to study plasminogen, we validated previous findings while suggesting a potentially novel relationship between plasmin(ogen)uria and estimated glomerular filtration rate (eGFR). Together, these findings suggest a role for plasmin(ogen) in mediating glomerular injury and as a viable targetable biomarker for podocyte-sparing treatments.

Keywords

glomerular; podocytes; plasminogen; amiloride; edema; eGFR

INTRODUCTION

Clinically it is often unclear whether progression of glomerular injury is driven strictly by the persistence of the initial insult and/or by a superimposed injurious process, a so-called “second hit”(1, 2). Human and experimental rodent studies have demonstrated that persistent non-selective proteinuria is a major risk factor of chronic kidney disease (CKD) progression and is accompanied by abnormal excretion of urinary serine proteases of which plasminogen/plasmin is a principal component (3–7). Prior studies of urinary plasminogen/plasmin—termed plasmin(ogen)uria—have focused on its activation of the amiloride-sensitive sodium channel ENaC in the distal nephron (8–12). In these studies, the degree of plasmin(ogen)uria was shown to be clinically associated with volume overload in patients with CKD of diverse etiologies, though most reports have concentrated on a limited number of subjects and/or diagnoses (3, 4, 13–16). However, clinical studies have yet to evaluate whether chronic aberrant filtration of plasminogen may also act proximally at the level of the glomerulus by promoting injury to podocytes, critical cells that help maintain the permeability and selectivity of the filtration barrier (17–22).

In our prior work with cultured human podocytes, we reported that podocytes express key mediators of the plasmin(ogen) system—urokinase plasminogen activator (uPA), uPA receptor (uPAR), tissue plasminogen activator (tPA)—and the transmembrane plasminogen receptor (PLG-RKT), which was previously shown to co-localize with uPA and facilitate the activation of plasminogen to plasmin by securing it to the cell membrane (23). After plasmin is cell bound, it is protected from inactivation by α2-antiplasmin, which leads to enhanced pericellular plasmin concentrations (24–26). Once activated, plasmin has pleiotropic actions. Through its proteolytic activity, plasmin promotes anoikis, a form of cell death induced by cell detachment from the extracellular membrane (24). Alternatively, plasmin can stimulate cellular cytokines and ROS production through non-proteolytic effects (27).

In our studies with cultured podocytes, we showed for the first time that plasminogen, after podocyte binding and conversion to plasmin, induces oxidative stress-mediated podocyte

injury directly by activation of NADPH oxidase 2/4 (NOX2/4) and the scavenger receptor CD36 (23). Of note, the ENaC inhibitor amiloride, with well-established off target effects inhibiting uPA-mediated conversion of plasminogen to plasmin (6, 28, 29) arrested the progression of plasmin(ogen)-mediated podocyte injury *in vitro* (23*).* Based on these overall findings, we postulated that in proteinuric subjects, excessive trans-glomerular plasminogen is converted to plasmin by the podocyte uPA-PLG-RKT system and that persistent exposure to plasmin serves as a "second hit" in glomerular diseases driving CKD progression independent of the initial insult.

To begin to address this hypothesis with 'proof of principle' studies, we explored *in vitro* podocyte morphologic changes induced by plasminogen treatment and tested the role of plasminogen in puromycin aminonucleoside (PAN) nephropathy, a well-established *in vivo* model of podocyte injury. Additionally, we have investigated cross-sectional associations between plasmin(ogen)uria and renal disease characteristics in one of the largest cohorts of glomerular diseases to study plasmin(ogen). Overall, we present strong supportive evidence for a causative role of the plasmin(ogen)-system in podocyte injury, with amiloride having glomerular protective properties. Our findings also advance clinical correlations of plasmin(ogen)uria as a biomarker of glomerular injury in proteinuric patients. Importantly, given such a function, the plasmin(ogen) pathway represents an attractive target for the development of mechanistic-based novel therapeutic interventions.

MATERIALS AND METHODS

Human podocyte culture

Human podocytes initially generated by MA Saleem Children's Renal Unit and Academic Renal Unit, University of Bristol, UK (30) were generously provided by S. Merscher and A. Fornoni, University of Miami. Briefly, human podocytes were cultured and differentiated in RPMI 1640 culture medium containing 10% FBS, 1% penicillin/streptomycin and 1% ITS, (30). The immortalized normal human podocytes were propagated at 33°C and then thermoshifted for differentiation for 10–14 days at 37°C. Terminally differentiated podocytes were starved in RPMI 1640 medium containing 0.2% FBS, 1% penicillin/ streptomycin and 1% ITS overnight before the experiments were performed. Recombinant human plasminogen was obtained from Enzyme Research Laboratories. Amiloride was purchased from MilliporeSigma.

Total Internal Reflectance Fluorescence (TIRF) Microscopy

TIRF microscopy was performed as previously described (31) to obtain a detailed assessment of focal adhesion parameters using a Leica DMi8 Infinity TIRF microscope and LASX (v.3.6). Focal adhesion morphometrics were assessed using immunofluorescence staining of paxillin (mouse anti-paxillin, BD Biosciences, Cat /# 610620), with an evanescent field depth of 75 nm, imaged under PBS supplemented with ProLong Live antifade agent (Thermo Fisher; Cat #P36975) using a 1.4NA Leica 63X oil TIRF objective at 30°C. To determine per cell morphometric values, simultaneous widefield images of the actin cytoskeleton and nuclei were obtained using Rhodamine-phalloidin (Thermo Fisher; Cat #R415) and DAPI (MD Biomedicals, Cat #0215757401), respectively. Separate

widefield images captured with a 0.5NA 20X air objective were used to determine cellular and nuclear size. All images were systematically processed in an unbiased, blinded, semiautomated manner and quantitatively analyzed following our high-content image analysis (HCA) segmentation guidelines outlined previously (32).

Animal studies

PAN nephropathy was induced as per established protocols (33, 34). Briefly, 6–8 weeks old male Wistar rats were intravenously injected either with either PAN (n=7; single IV 100 mg/kg) or PAN + amiloride (n=8; PAN + 0.5mmol/L amiloride). PBS-injected age-matched rats (n=6) served as controls. Amiloride was administered in drinking water starting at the time of PAN or PBS injection. Animals were euthanized 7–8 days after PAN injection. Kidneys were removed and glomeruli were isolated by serial sieving in ice-cold PBS. All animal protocols were approved by IACUC at the Icahn School of Medicine at Mount Sinai and the Miller School of Medicine at University of Miami. Morphometric/histologic analyses were performed by investigators blinded to experimental group allocation.

Study participants

Subjects with glomerular disease biopsy-proven diagnoses (diabetic kidney disease, IgA, membranous nephropathy; lupus nephritis, FSGS, arterionephrosclerosis) were recruited and provided written informed consent at enrollment, as per the approved Institutional Review Board (IRB). Freshly voided urine samples were collected and spun at 3000 rpm for 15 minutes. Supernatants were aliquoted and stored at −80°C prior to use. All clinical data was collected from electronic medical records. Frozen sections from archived human biopsy material were obtained from the Icahn School of Medicine at Mount Sinai under an IRB approved protocol. All biopsies were clinically indicated, and only extra tissue not required for diagnostic purposes was permitted for research use.

Urinary Biomarker Determination

Rat urine plasmin(ogen) were measured using commercial kits (E-25PMG; ICLLAB, Portland, OR, USA), as per protocol. Rat urine creatinine was measured according to the manufacturer's protocol (Item No: 500701; Cayman Chemicals). Total rat urine protein was determined using BioRad Protein Assay reagents (Item Nos: 500–0113, 500–0114, 500–0115). For human samples, plasmin(ogen) from urine at time of biopsy was measured using a commercial kit (IHPLGKT-TOT; Innovative Research, Novi, MI, USA), as per protocol. Concentrations were estimated from a standard curve and normalized by urine creatinine. All other patient values including creatinine were determined by the chemistry laboratory at Icahn School of Medicine at Mount Sinai.

Western blotting, Immunofluorescence, Immunohistochemistry

For immunoblotting, samples were processed by standard protocol. Briefly, tissue was homogenized and protein quantified by Bradford assay. Equal amounts of protein were loaded and separated by SDS-PAGE and transferred to nitrocellulose. Data were normalized by β-actin and expressed as fold-change relative to control. Anti-synpo was a gift from Dr. Peter Mundel (MGH, Boston, MA, USA), all other western blot primary antibodies were

purchased from Santa Cruz (Dallas, TX, USA). For immunostaining, sections were cut on slides which were blocked and incubated with the appropriate primary and secondary antibodies, as per standard protocol. Secondary Alexa conjugated antibodies were purchased from Thermo Fisher (Life Technologies, Carlsbad, CA, USA). Nuclei were labeled with DAPI. Anti-Paxillin was purchased from ThermoFisher; anti-CD36 from Santa Cruz; anti-8-Oxo-G from Abcam (Cambridge, MA). Standard DAB IHC was performed at Mount Sinai Department of Pathology, using Ventana Discovery Ultra IHC/ISH research platform. Antigen retrieval was achieved with proprietary CC1 solution (Roche Diagnostics, Indianapolis, IN, USA) for 1 hr. Primary antibody against plasmin(ogen), which was diluted in PBS 1:400, was manually applied and incubated for 1 hr, followed by secondary anti-rabbit antibody (Roche Diagnostics). Counterstain was DAB.

Proteomics

Isobaric labelled shotgun proteomics was performed as previously described.(33) Briefly, samples were lysed in urea with protease and phosphatase inhibitors and proteins were tryptically digested overnight. Peptides were labeled using iTRAQ isobaric tags (AB Sciex, Framingham, MA, USA) per manufacturer's instructions and combined peptides were desalted and fractionated. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was performed using an UltiMate 3000 LC System and an Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). MS/MS spectra were analyzed using Mascot and Sequest search engines with the Proteome Discoverer platform. A false discovery rate cutoff of 0.01 was used to limit the forward/reverse database searches that yielded peptide identification with a 95% confidence interval. Relative quantification was performed on the Scaffold platform using the log2 values of iTRAQ label ratios.

Clinical Outcomes and Statistical Analysis

The primary outcomes were edema status (yes/no) and eGFR at time of biopsy. Edema status was extracted from the notes in the EMR. All urinary markers (protein/creatinine, albumin/creatinine, plasminogen/creatinine) were log-transformed to approximate normal distribution. Correlations between log-transformed biomarkers and relevant characteristics were assessed using Spearman's partial correlations. Boxplots that denoted the median, IQR, minimum and maximum were used to visualize the distribution of each urinary biomarker with edema status yes vs. no and eGFR < 60 vs. ≥ 60. The association for each log-transformed biomarker with the kidney-related clinical characteristic was evaluated by univariable and multivariable logistic regression for edema status and with univariable and multivariable linear regression for eGFR (adjusted for age, gender, race/ethnicity, RASi or diuretic use, and eGFR, as appropriate) using SAS® University Edition (Cary, NC).

RESULTS

Plasminogen reduces podocyte focal adhesion marker expression

To further define the cell biological consequences of plasminogen-induced podocyte injury, we treated immortalized human podocytes with plasminogen versus control and performed F-actin (phalloidin), focal adhesion (paxillin), and nuclear (DAPI) staining followed by confocal microscopy. We found that plasminogen treatment (1μM) reduced podocyte focal

adhesion marker expression (Figure 1). Conversely, focal adhesion marker expression was preserved with treatment of podocytes with plasminogen and amiloride together (Figure 1). Notably podocyte F-actin expression was not reduced by plasminogen treatment. We then utilized HCA of TIRF images to perform a detailed assessment of focal adhesion parameters of human podocytes that are treated with plasminogen or those co-treated with plasminogen plus amiloride for 24 hours. HCA shows that plasminogen treated podocytes have significantly fewer focal adhesions with larger surface area, perimeter and aspect ratio (i.e., elongation). Partial rescue of focal adhesion area and perimeter (but not number or elongation) was conferred by amiloride co-treatment (Figure 2). The alterations in focal adhesion morphology was also reflected in cell and nuclear spreading area, which were both significantly reduced with plasminogen treatment.

Amiloride decreases proteinuria in PAN nephropathy

We next sought to extend our hypothesis to an *in vivo* model. The PAN nephropathy model of podocyte injury has been previously shown to be associated with increased urinary plasmin(ogen) (10). To reproduce these findings and determine the effects of amiloride treatment, adult male Wistar rats (6–8 weeks old) were treated with a single intravenous dose of PAN (100 mg/kg), PAN plus amiloride (0.5 mmol/L), or vehicle, and sacrificed on day eight. PAN treated rats developed increased proteinuria compared to controls (6.84±0.62 mg/mg·Cr vs. 0.68±0.07 mg/mg·Cr; $p<0.0001$). Treatment with amiloride was associated with a 54.5% reduction in proteinuria, as compared to PAN alone (3.11±0.32 mg/mg·Cr; $p<0.0001$) (Figure 3A). In addition, PAN treatment caused increases in plasmin(ogen)uria (15.96±2.47 μg/mg·Cr vs. 2.16±0.49 μg/mg·Cr; $p<0.0001$) (Figure 3B). Amiloride reduced PAN-induced plasmin(ogen)uria by 22.0%, though the difference was not statistically significant ($p=0.095$) (Figure 3B). Thus, PAN nephropathy is associated with increased proteinuria and urinary plasmin(ogen), both reduced by amiloride.

Glomerular plasmin(ogen) is increased in PAN nephropathy

Given the reversibility of PAN nephropathy induced by a single injection (35–38), a separate time course experiment was conducted in order to objectively trend the time-dependent nature of glomerular plasmin(ogen). Across two proteomic analyses, the average size of the top 50 enriched proteins by mass-spectrometry were 85±56 kDa and 59±40 kDa, respectively, suggesting a wide range of identified molecular weight proteins. High plasmin(ogen) levels were quantified via iTRAQ proteomics in glomeruli of PAN-treated rats—glomerular plasminogen was elevated on day 3 and peaked on day 7 before returning to baseline by day 14–21 (Figure 4A). The peptides identified as plasminogen were from both the N- and C-terminal regions and were not specific to uncleaved plasminogen or plasmin (data not shown). We confirmed the presence of elevated levels of plasmin(ogen) within glomerular isolates by western blotting (Figure 4B). Taken together, excessive filtration of plasminogen occurs early following PAN-mediated podocyte injury, whereupon plasmin(ogen) strongly binds within the glomerulus.

Amiloride treatment decreases oxidative stress and improves podocyte integrity in PAN nephropathy

We previously demonstrated that plasmin(ogen)-mediated ROS generation involved podocyte-specific upregulation of NADPH oxidases NOX2/4 and the B scavenger receptor CD36 (23). Here, we sought to determine the oxidative stress changes associated with PAN treatment with or without amiloride. By immunofluorescence staining, we found that PAN treatment increased glomerular CD36, which colocalized with the podocyte marker synaptopodin (synpo) (Figure 5A). Glomerular gene expression by qPCR also showed increased CD36 with PAN (data not shown). Similarly, we showed enhanced double labeling immunofluorescence for 8-OxoG and synaptopodin, supporting PAN-mediated reactive oxygen species (ROS) generation in podocytes (Figure 5B). Importantly, PAN-induced increases in both CD36 and 8-oxo-G were ameliorated by amiloride treatment (Figures 5A-B).

Studies by us and others have highlighted the contribution of podocyte apoptosis to glomerular disease progression (21–23). In support of our previous study, which showed plasmin(ogen)-mediated podocyte apoptosis augmented by oxidized low-density lipoprotein (oxLDL), we found that total glomerular caspase-3 expression was enhanced with PAN treatment and reduced with amiloride (Figure 5C). Moreover, we determined that glomerular levels of markers of podocyte integrity, specifically nephrin and synaptopodin, were reduced in PAN nephropathy (Figure 5C). Of note, both nephrin and synaptopodin are sensitive to ROS mediated injury (39, 40). Overall, the pathophysiologic changes observed with PAN treatment were either abrogated or reduced with amiloride (Figure 5). Taken together, these findings suggest that amiloride is protective/stabilizing against PAN-induced oxidative stress and glomerular injury.

Urinary plasmin(ogen) excretion is increased in human glomerular disease

Our study cohort included 128 patients with glomerular diseases with urine samples collected at the time of kidney biopsy. The full characteristics of the cohort are summarized in Table 1. Subjects had an average age of 48.3 years with mean eGFR 57.5 ± 36.7 ml/min/1.73 m^2. Of the subjects, 27% were edematous with 32% and 47% prescribed diuretics and angiotensin converting enzyme inhibitor (ACEi) or angiotensin II receptor blockers (ARB) respectively, at the time of biopsy. Participants with urinary plasmin(ogen) (log μg/mg•Cr) in the upper quantile tended to be older with lower eGFR and were more likely to have edema with a prescription for diuretics and ACEi/ARB (Table 1).

Urinary plasmin(ogen) has been previously shown to be associated with hypervolemia in limited patient cohorts (3, 4, 13–16). To validate these findings across a larger sample size in the setting of biopsy-proven glomerular diseases, we sought to define these relationships within our cohort. At baseline across all subjects, plasmin(ogen)uria (log μg/mg•Cr) was strongly positively correlated with both proteinuria (log mg/mg•Cr; $r=0.8348$, $p<0.0001$) and albuminuria (log mg/mg•Cr; $r=0.8219$, $p<0.0001$) (Figure 6). In addition, plasmin(ogen)uria had an inverse correlation with eGFR ($r=-0.3320$, $p=0.0001$) (Figure 6). To examine whether relative urinary plasmin(ogen) was associated with edema status and kidney function, we compared plasmin(ogen)uria with cross-sectionally data. Higher levels of all

three biomarkers—plasmin(ogen)uria, albuminuria, and proteinuria—were associated with the presence of edema. However, only plasmin(ogen)uria was higher in patients with eGFR < 60 ml/min/1.73 m^2, compared those with eGFR ≥ 60 ml/min/1.73 m^2 (Figure 6).

In univariable analyses, plasmin(ogen)uria, along with both albuminuria and proteinuria, were similarly associated with increased odds of edema at time of biopsy (Table 2). Adjusting for age, gender, race, use of ACEi/ARB or diuretics, and baseline eGFR did not affect the observed strengths of association. The three biomarkers had similar negative correlations with eGFR at time of biopsy, all of which were modestly attenuated after adjustment for covariates. (Table 2).

Glomerular plasmin(ogen) is increased in human FSGS

Given our results demonstrating glomerular plasmin(ogen) in the PAN nephropathy rodent model, we sought to translate these novel findings to the clinical setting. Consistent with our observations in PAN-treated rats, we observed increased plasmin(ogen) expression within glomeruli from a series of human kidney biopsies of patients with FSGS, as compared to age-matched normal control kidney tissue (Figure 7).

DISCUSSION

In the present study, (i) we experimentally assessed whether our previously published *in vitro* results could be translated to a well-established *in vivo* proteinuric rodent model and (ii) sought to better define the associations between plasmin(ogen)uria and clinically relevant kidney disease characteristics in patients with proteinuric glomerulopathies. Collectively, our novel findings, together with the previous *in vitro* data, lends further support to a model in which urinary plasmin(ogen) acts a “second hit” with direct and/or indirect effects on podocytes and glomerular integrity in promoting CKD progression.

Independent of baseline eGFR or etiology, total proteinuria is a marker of kidney damage and a strong predictor of progression to end-stage renal disease (41–48). As shown in the SPRINT trial, proteinuric patients who reach CKD stage 3–4 relentlessly progress to ESRD in spite of intensive blood pressure control (49, 50). Moreover, increasing proteinuria in the setting of CKD correlates with increased risk of cardiovascular disease and all-cause mortality (43–48).

Few reports, however, have identified a role for specific filtered proteins that contribute to kidney damage through cytotoxic effects on podocytes as “second hits” (1, 2). Such work includes the identification of albumin as well as albumin bound FFA as a potential contributors to both glomerulosclerosis and tubulointerstitial injury via direct and indirect mechanisms (51–54).

In our studies with cultured human podocytes, we demonstrated the expression of PLG-RKT, uPA, and uPAR, which together facilitate binding and activation of plasminogen to plasmin on the cell surface (23). Moreover, we observed that podocyte-bound plasmin induces oxidative stress and, importantly, that amiloride, a known potent inhibitor of uPA, rescued the plasminogen-induced injury response (23). Here we have shown that

plasminogen treatment directly reduced focal adhesion marker expression. To expand upon the hypothesis that filtered plasminogen serves as a “second hit” in glomerular damage, we induced PAN nephrosis in rats, a model in which ROS-mediated cytoskeletal dysfunction has been well documented to promote foot process damage (23, 35, 55, 56). PAN-treated rats developed severe proteinuria and plasmin(ogen)uria, similar to our studies in rodent models of FSGS and of HIV-associated nephropathy (10, 23).

Mechanistically, and in support of our *in vitro* data, we found increased markers of apoptosis and oxidative stress, including CD36, 8-oxo-G and caspase-3, in glomerular isolates from PAN-treated rats, due at least in part to upregulation of NOX2/4 activity (23). PAN treatment was also associated with a reduction of glomerular levels of synaptopodin and nephrin, two critical regulators of podocyte functional and structural homeostasis (57–64). Furthermore we determined—for the first time to our knowledge—that there was increased plasmin(ogen) within glomeruli of PAN rats and, remarkably, also in kidney biopsies of proteinuric FSGS patients, further supporting a model in which plasmin(ogen) is capable of *in vivo* podocyte binding and mediating *in situ* injurious effects.

Following amiloride treatment in PAN rats, we observed declines in proteinuria with a trend toward a decrease in plasmin(ogen)uria. Of note, utilizing a plasmin specific detection method, Svenningsen et al. reported that in the presence of amiloride, urinary plasmin(ogen) is predominately unconverted plasminogen, in line with its function as an inhibitor of plasminogen cleavage/conversion to plasmin (10). Given that current commercially available immunoassays do not discriminate between plasminogen and plasmin, we are unable to specifically report upon the relative urinary plasmin/plasminogen ratio.

The rescue phenotype observed with amiloride was consistently observed throughout our study across multiple downstream targets, as seen by both immunofluorescence and western blotting. While cell and nuclear spreading area were significantly ameliorated by amiloride co-treatment, we noted that there was no significant rescue for some of the focal adhesion markers impacted by plasminogen. This is unsurprising since focal adhesion assembly and maintenance are complex processes that involve numerous signaling pathways, many of which may be differentially impacted by amiloride. Earlier reports on amiloride initially demonstrated that it potently inhibits urinary uPA activity, preventing plasmin-mediated stimulation of ENaC activity and sodium retention (4, 6, 10). In our previous studies with cultured human podocytes we showed that amiloride, as an inhibitor of the conversion of plasminogen to plasmin, prevented plasmin-generated ROS in podocytes, thereby averting oxidative stress-mediated podocyte injury (23). Our current data in PAN rats suggests that amiloride may have direct podocyte-sparing properties *in vivo,* upstream of its ENaC functionality. The findings are also consistent with other reports showing that amiloride has off-target anti-proteinuric effects by direct action on podocytes with the drug being shown to inhibit podocyte urokinase receptor expression (uPAR) (29, 65).

We have previously demonstrated high levels of plasmin(ogen)uria in a murine model of HIV nephropathy (23). In a distinct model of HIV-induced podocytopathy-associated proteinuria, Matsusaka et al showed that acute unilateral ureteral obstruction mechanically arrested GFR and the trans-glomerular protein passage underlying proteinuria. Importantly,

after one week, they observed preservation of podocyte morphology in the ipsilateral kidney, whereas proteinuria, progressive podocytopathy, and glomerulosclerosis developed in the contralateral unobstructed kidney (66). This finding fits with our hypothesis that prevention of podocyte exposure to plasmin(ogen), either mechanically with ureteral obstruction or biochemically through the inhibition of its conversion to plasmin, could plausibly provide podocyte protection in proteinuric glomerulopathies.

Taken together, these previous studies, in combination with our own *in vitro* and *in vivo* work, strongly support the hypothesis that plasmin(ogen) serves as a putative causative agent, or "second hit," in CKD progression, in addition to its known function as a biomarker and mediator in promoting volume overload. Studies in patients with CKD of various etiologies have both detected the presence of excessive urinary plasmin(ogen) and demonstrated its role as a novel biomarker (3, 4, 13–16). Plasmin(ogen)uria has been correlated with markers of glomerular proteinuria, namely albuminuria, and found to be associated with sequela of fluid overload, including hypervolemia and hypertension, though most studies have been limited by sample size and/or single-disease etiologies (13–15, 28).

In our cohort of 128 patients with biopsy-proven podocytopathies, we examined the association between urinary plasmin(ogen) levels and both volume overload (as determined by edema status) and kidney function (as determined by eGFR), compared with both albuminuria and proteinuria. The correlation we observed between plasmin(ogen)uria and albuminuria ($r=0.8219$) is consistent with previous reports. We also observed that urinary excretion of plasmin(ogen) was independently correlated with edema status at time of biopsy, as are both total proteinuria and albuminuria, with all urine markers exhibiting similar degrees of association. These cross-sectional results for edema at the time of biopsy are similar to previous findings from studies conducted later in the disease course, including in patients with stable CKD (4, 14, 15).

We observed inverse correlations between the three biomarkers and eGFR at biopsy. This represents a potential relationship between urinary plasmin(ogen) excretion and eGFR in the setting of multiple biopsy-proven glomerular diseases. Similar results have been observed specifically in the setting of lupus nephritis (67). Although the cross-sectional nature of our study prevents conclusions on a direct contribution of plasmin(ogen) to changes in eGFR, such a relationship is of interest, given the likely pro-injurious role of plasmin(ogen) on the glomerulus.

The results of this study should be viewed in light of some limitations. Our rodent model is associated with plasmin(ogen)uria, with amiloride having rescue effects that were previously attributed to plasmin(ogen) *in vitro* (23). Although other proteins and/or mechanisms may account for some of the observed results, our data provides indirect evidence for a role of plasmin(ogen) in mediating injury in the PAN model. Another limitation is that the cross-sectional nature of our study, absence of longitudinal outcomes and small sample size prevented us from performing a mediation analysis to test whether adding plasminogenuria increases the prediction odds ratio of albuminuria for the development of edema or decline in eGFR. The small sample size also precluded a ridge regression analysis for highly collinear plasminogenuria and albuminuria without very high penalties and bias (14).

However, the proposed biological mechanism, as shown by our *in vitro* and *in vivo* studies, supports a direct role plasmin(ogen). This remains one of the largest to study plasmin(ogen) to date across biopsy-proven etiologies and lays the foundation for further exploration of the relationship between plasmin(ogen)uria and eGFR longitudinally in a similar population with a larger sample size and time-to-event data.

Collectively, our results, in support of previous findings, suggest a pathogenic role for intra-glomerular plasminogen activation, in which *in situ* conversion of plasminogen to plasmin on podocyte surface by podocyte-bound uPA leads to plasmin-mediated podocyte injury and worsening of the underlying proteinuric glomerular process. Once cell bound, plasmin is protected from inactivation by inhibitors, such as α−2-antiplasmin, and is able to initiate cell responses linked to both its proteolytic and non-proteolytic actions, including degradation of matrix proteins facilitating podocyte detachment and induction of CD36 and ROS synthesis resulting in mitochondrial injury and apoptosis. In combination with our clinical findings, this suggest a dual function of plasmin(ogen) in the progression of kidney disease as a targetable biomarker, with effects at two independent sites within the nephron. As such, this may signal a shift in the current paradigm from the view of targeting plasmin(ogen)uria to solely treat sodium retention in nephrotic syndrome to early inhibition of plasmin activity in promote glomerular integrity in a wide-range of podocytopathies.

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ABBREVIATIONS

8-OxoG	8-oxoguanine
ACEi	ACE inhibitor
ARB	angiotensin II receptor blockers
AMIL	amiloride
CASP3	caspase 3
CD36	cluster of differentiation 36
CKD	chronic kidney disease
Cr	creatinine
eGFR	estimated glomerular filtration rate
eNaC	amiloride-sensitive sodium channel

ESRD	end stage renal disease
FSGS	focal segmental glomerulosclerosis
HIVAN	HIV-associated nephropathy
iTRAQ	isobaric tags for relative and absolute quantitation
NOX2/4	NADPH oxidase 2/4
NPHS1	nephrin
oxLDL	oxidized low-density lipoprotein
PAN	puromycin aminonucleoside
PLG-RKT	plasminogen receptor with a C-terminal lysine
RASi	renin-angiotensin system inhibitors
ROS	reactive oxygen species
SYNPO	synaptopodin
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
uPAR	uPA receptor

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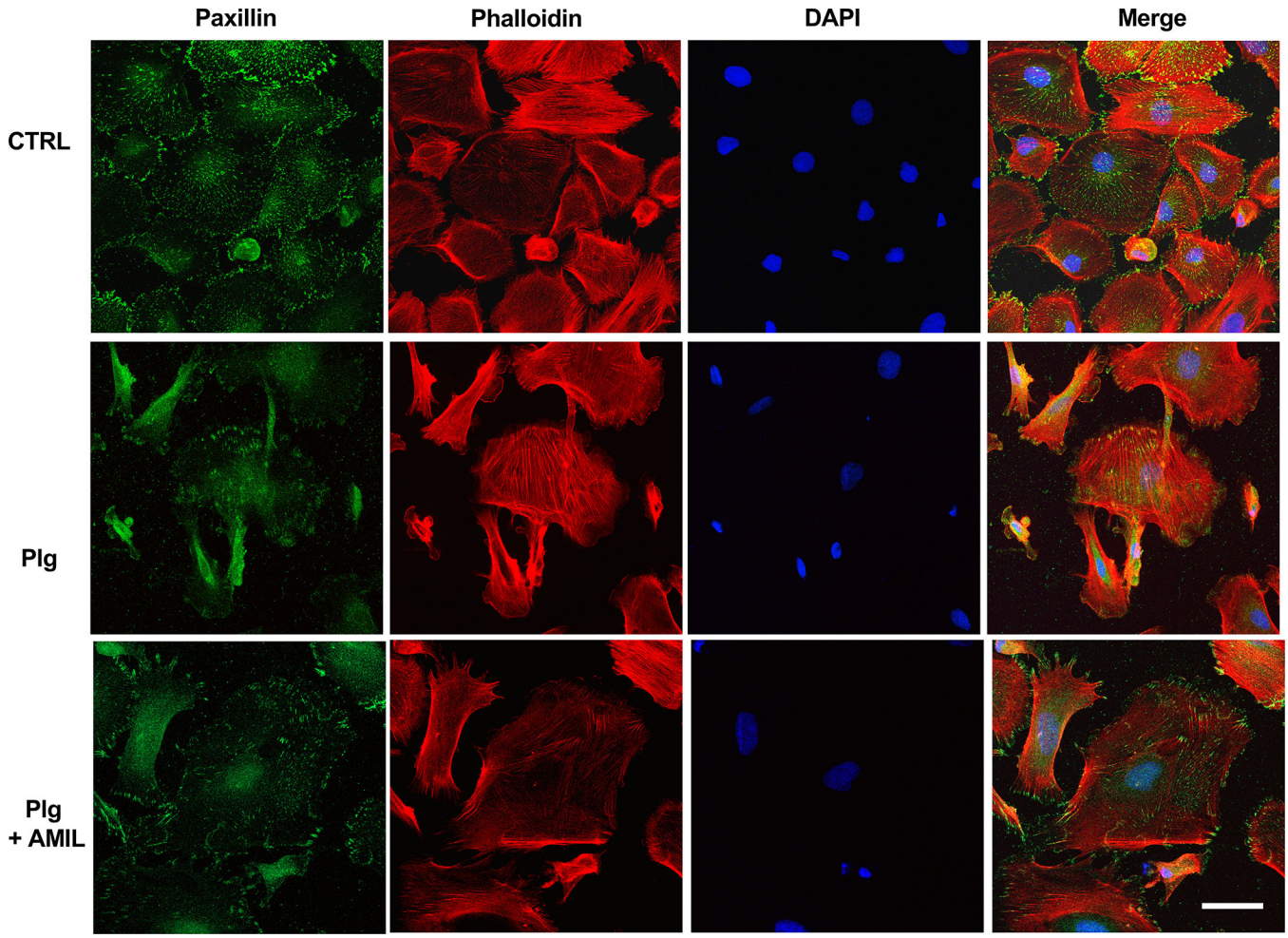

Figure 1: Amiloride protection from plasminogen-induced morphologic changes.

Plasminogen treatment (1μM, 24 hrs) decreases focal adhesion marker (paxillin) expression with rescue noted by amiloride co-treatment (10 μM). Plasminogen treatment did not affect F-actin (phalloidin) expression. Scale bar = 50 μm

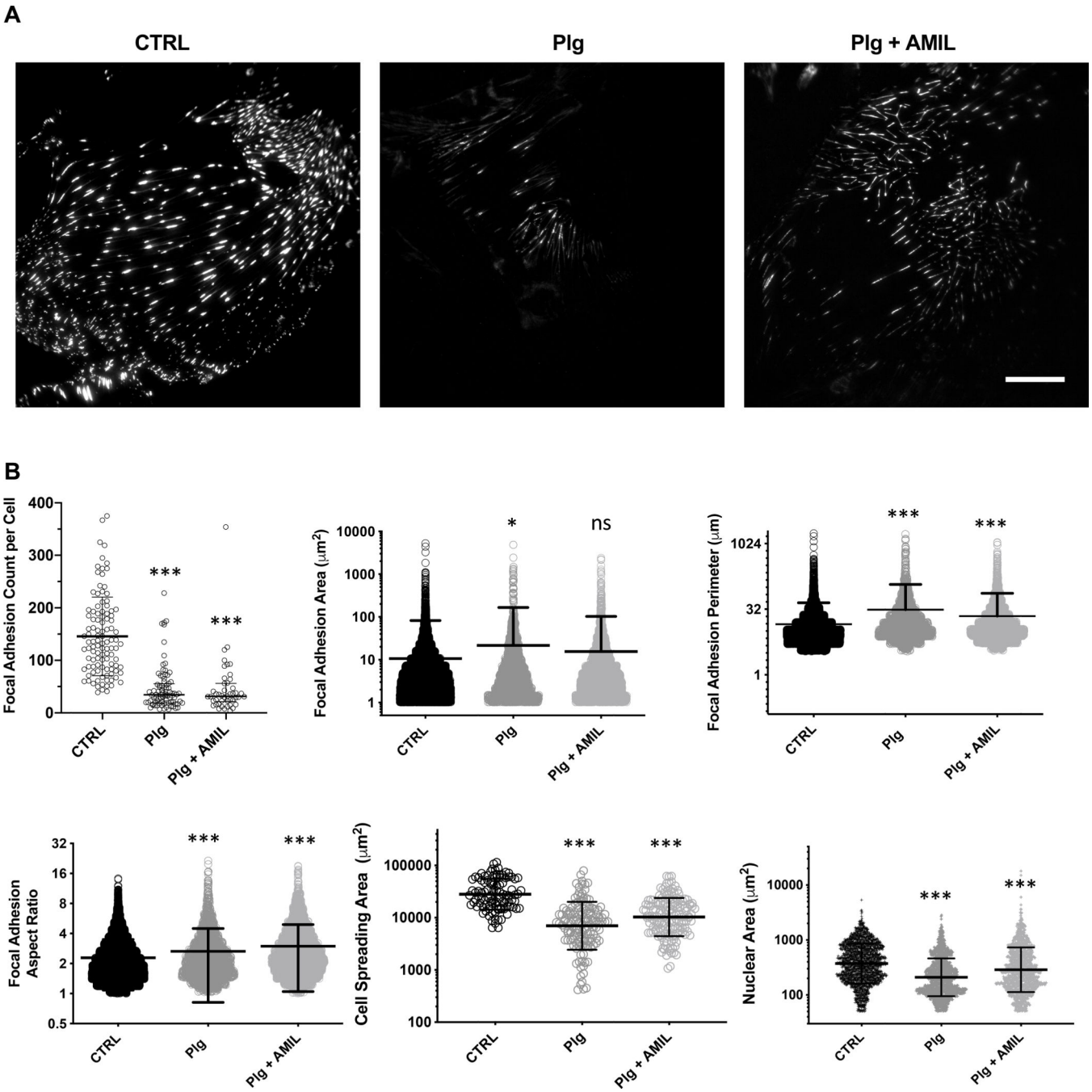

Figure 2:

Podocyte morphologic changes with Plasminogen treatment (A) Representative total internal reflection fluorescence (TIRF) images of untreated (CTRL), plasminogen treated (Plg) and plasminogen-amiloride co-treated (Plg + AMIL) human podocytes imaged with an evanescent field depth of 75 nm. (B) Quantitative high content image analysis (HCA) showed that Plg treatment resulted in reduction of focal adhesion count per cell, nuclear area and cell spreading area with increases in focal adhesion area, perimeter and aspect ratio.. Co-treatment with Plg + AMIL partially rescued podocytes from these morphologic

changes. *P<0.05, ***P< 0.0005 relative to CTRL using a Kruskal-Wallis test. Scale bar = 50 μm

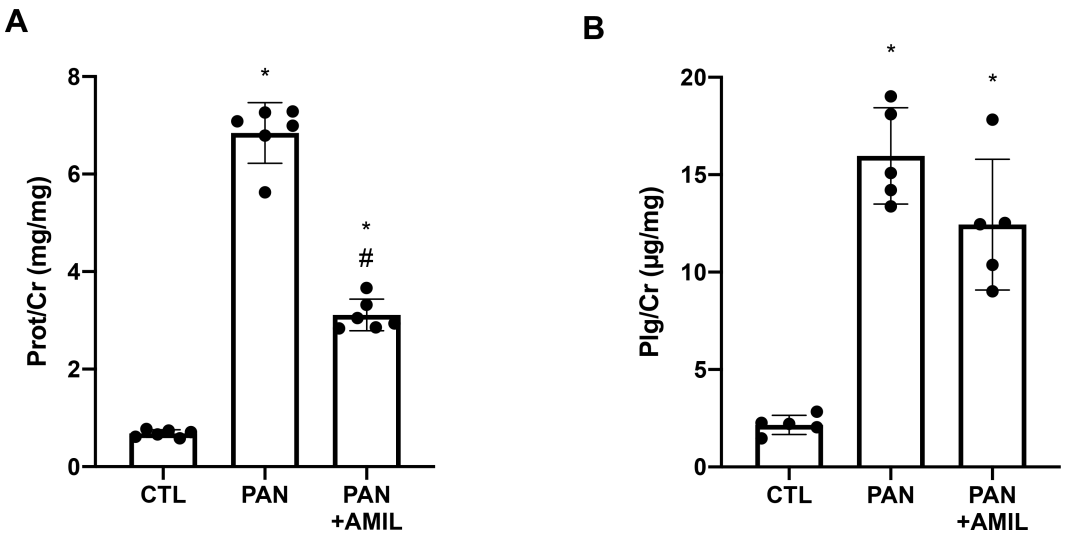

Figure 3: PAN nephropathy promotes urinary excretion of total protein and plasmin(ogen).

PAN caused increased (**A**) proteinuria and (**B**) plasmin(ogen)uria, which were decreased by administration of amiloride (0.5mmol/L amiloride q.d.). Plg/Cr, urinary plasmin(ogen)/creatinine; Prot/Cr, urinary protein/creatinine; amil, amiloride; * $p<0.05$ relative to CTL; # $p< 0.05$ relative to PAN alone; ANOVA.

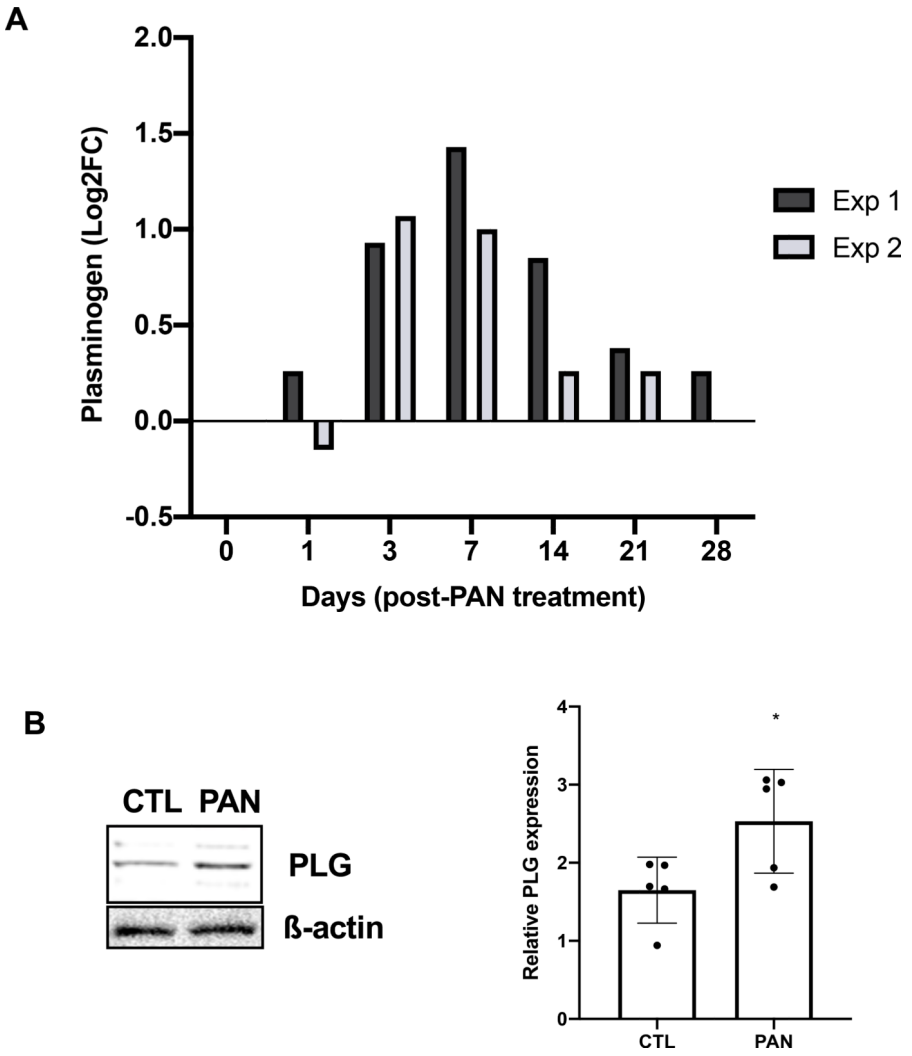

Figure 4: PAN nephropathy increases glomerular plasmin(ogen).

Glomeruli from PAN treated rats were isolated for mass spectrometry or immunoblotting. (**a**) Plasmin(ogen) levels increased early following single dose PAN treatment by proteomics; dark gray box, experiment 1; light gray box, experiment 2. Plasmin(ogen) levels were increased in glomeruli of PAN-treated rats as detected by western blotting (**b**). Representative images are shown. The abundance of β-actin was used to normalize and densitometric analysis was performed using ImageJ. Each bar represents the mean ± SD across 4–5 rats. PLG, plasmin(ogen); * $p < 0.05$; t-test.

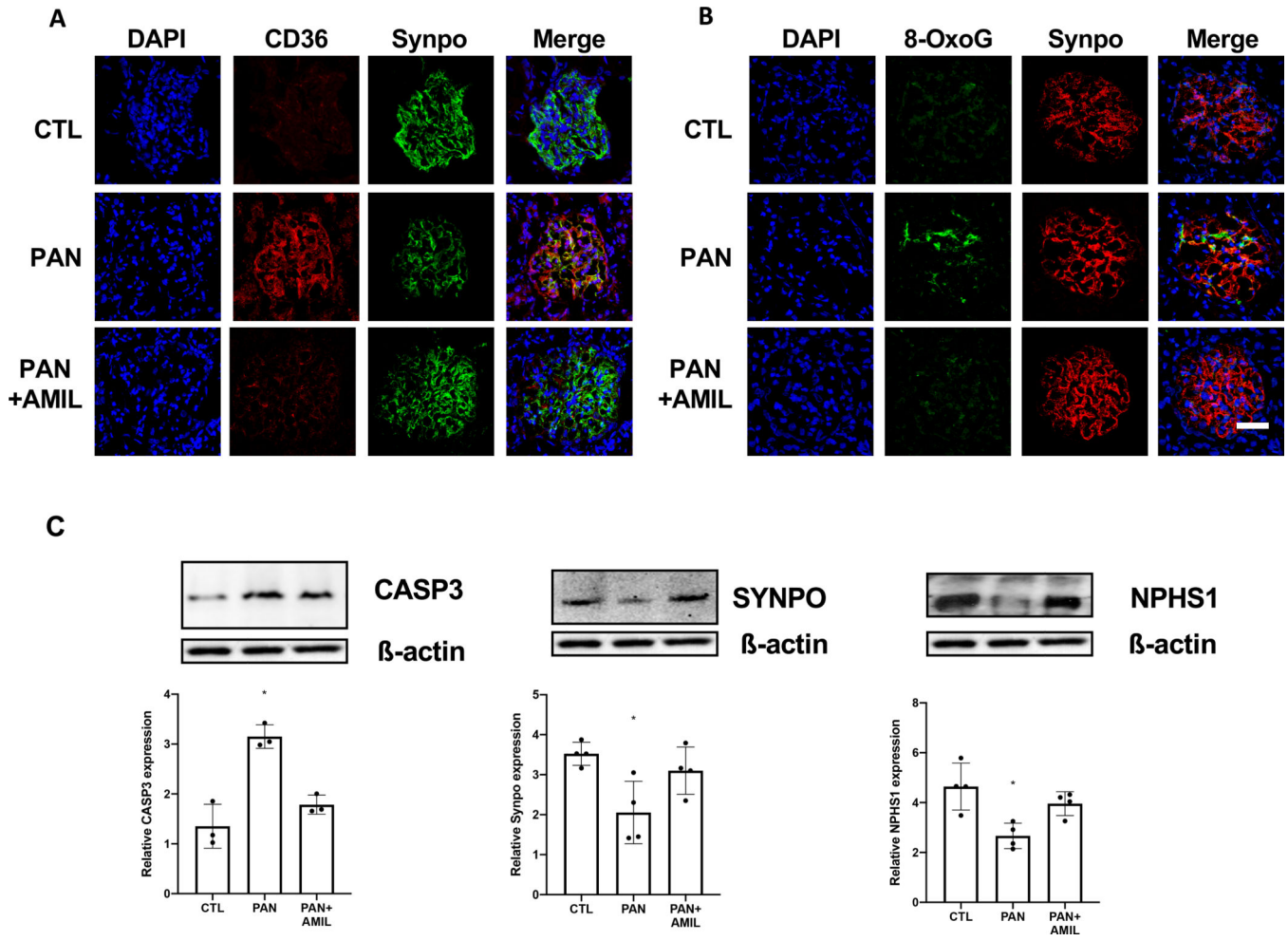

Figure 5: PAN nephropathy is associated with increased oxidative stress and impaired podocyte homoeostasis

(**a**, **b**) CD36 and 8-OxoG robustly accumulated in podocytes following PAN treatment. Administration of amiloride caused dramatic reduction in both markers. Red color shows staining for CD36 (**a**) and the podocyte marker synaptopodin (**b**). Green color shows staining for synaptopodin (**a**) and 8-Oxo-G (**b**). Nuclei were colored blue with DAPI. (**c**) Significant bands for caspase 3, synaptopodin, and nephrin were observed in samples of isolated glomeruli from PAN-treated rats. Representative images are shown. The abundance of β-actin was used to normalize and densitometric analysis was performed using ImageJ. Each bar represents the mean ± SD across 3–4 rats. * $p<0.05$. ANOVA. Scale bar = 50 μm

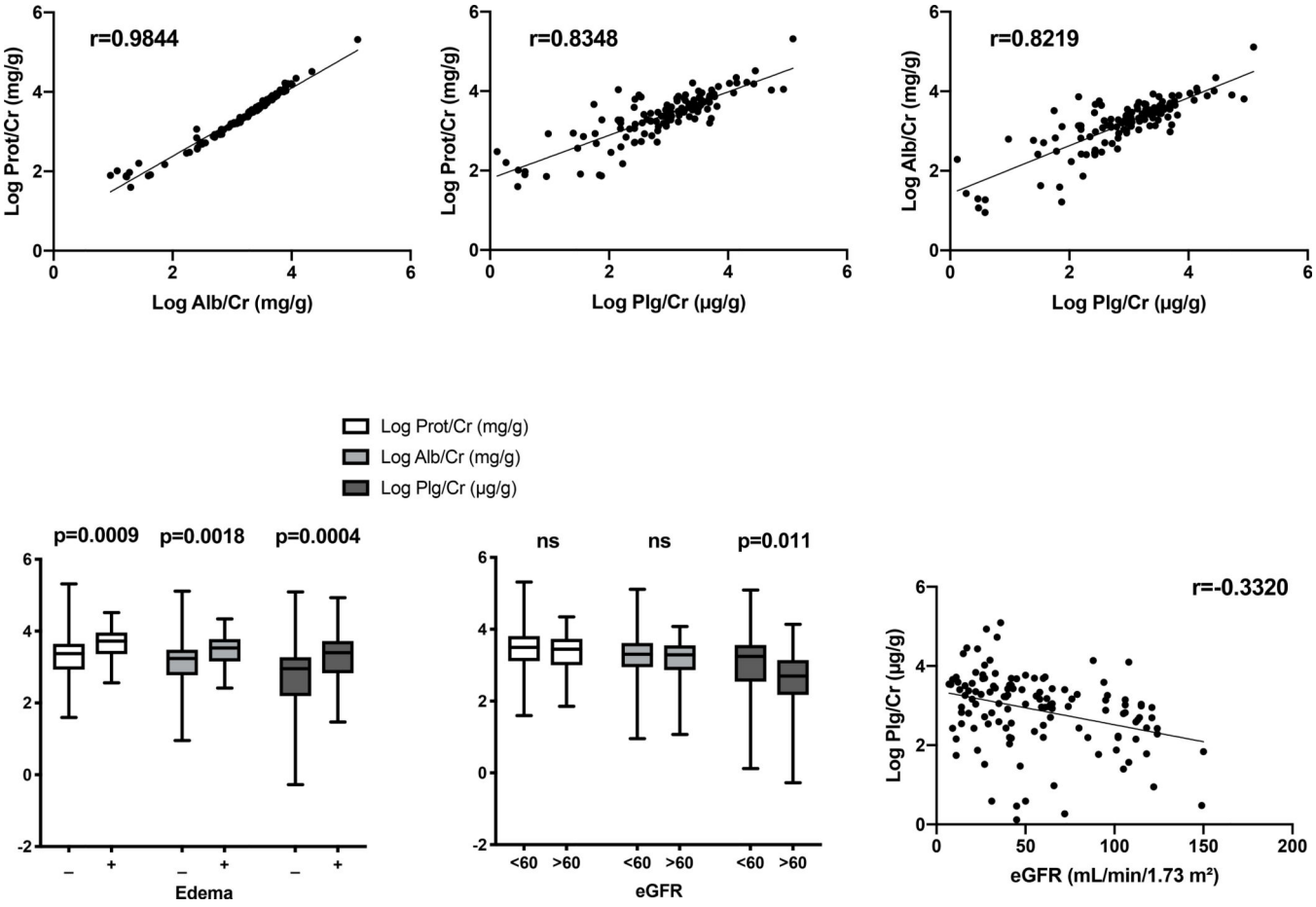

Figure 6: Plasmin(ogen)uria correlates with urinary biomarkers and kidney disease outcomes in cross-sectional analysis of patient urines.

Log of urinary plasmin(ogen)/creatinine (log Plg/Cr) correlated with both total urine protein/creatinine (log Prot/Cr) and albumin/creatinine (log Alb/Cr) at biospy. The presence of edema was significantly associated with higher levels of all tested urine markers whereas high log Plgl/Cr correlated weakly with lower eGFR at time of biopsy. The ordinate represents log units, but the units for log Plg/Cr (µg/g) differ from those of log Prot/Cr and log Alb/Cr (mg/g). Whiskers represent standard deviations. T-test. White boxes, log Prot/Cr; light gray boxes, log Alb/Cr; dark gray boxes, log Plg/Cr

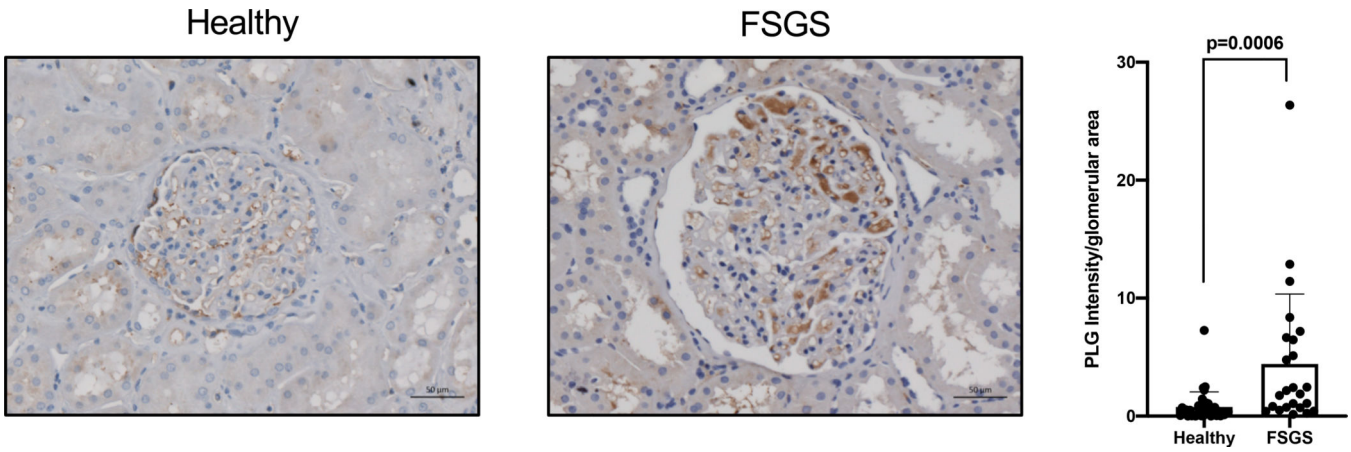

Figure 7: Immunohistochemistry of kidney from patients with focal segmental glomerulosclerosis (FSGS) is consistent with glomerular-associated plasmin(ogen)

(a) Immunostaining for plasmin(ogen) was performed on paraffin-embedded human kidney sections. Representative images are shown. (b) Quantification of glomerular staining intensity was calculated and is shown as a fold change relative to control samples. Glomerular plasmin(ogen) staining is significantly increased in patients with FSGS compared with control subjects. Total number of glomeruli analyzed: n = 37 control (9–14 glomeruli from each of 3 individual patients), n = 24 FSGS (4–10 glomeruli from each of 3 individual patients); t-test. Scale bar = 50 μm

Table 1 |

Study characteristics of glomerular disease patient cohort. Values reported are n (percentages) for categorical variables and means for continuous variables. Data are presented as means stratified by Plg/Cr quantile based on median cohort value. eGFR is the GFR determined using the CKD-EPI formula. Alb/Cr, urinary albumin/creatinine; Prot/Cr, urinary protein/creatinine; Plg/Cr, urine plasmin(ogen)/creatinine

	log Plg/Cr (μg/g)			
	Summary data	**Lower quantile**	**Upper quantile**	**p**
Subjects	128	63	65	–
Age, yr	48.3	44.3	52.2	0.0032
Sex, % Female	50.7	55.6	46.2	0.2875
Race, % Hispanic	29.7	25.4	33.8	0.2956
Log Alb/Cr, mg/g	3.15	2.7	3.57	<0.0001
Log Prot/Cr, mg/g	3.35	2.94	3.75	<0.0001
eGFR, mL/min/1.73 m^2	57.5	71	44.5	0.0001
Edema, % subject	27.3	15.9	38.5	0.0042
Diuretic use, % subject	32.0	23.8	40	0.0497
ACEi/ARB use, % subject	46.9	34.9	58.5	0.0076

Table 2 |

Urine biomarkers are associated with edema and eGFR at time of biopsy. (A) All three markers tested (log Prot/Cr, log Alb/Cr, log Plg/Cr) correlated with increased odds of edema at biopsy in both univariate and adjusted regression models. (B) All three markers tested (log Prot/Cr, log Alb/Cr, log Plg/Cr) were associated with reduced eGFR in both univariate and adjusted regression models. Negative values represent decrements in eGFR in log change mg/g or μg/g as indicated.

A

Predictor	**Edema** OR (95% CI); p value	
	Univariate	Multivariable[a]
Proteinuria, log mg/g Cr	3.81 (1.74 to 9.73); p=0.0022	3.80 (1.58 to 10.66); p=0.0056
Albuminuria, log mg/g Cr	3.43 (1.61 to 8.75); p=0.0041	3.49 (1.50 to 9.66); p=0.0079
Plasmin(ogen)uria, log μg/g Cr	2.58 (1.53 to 4.79); p=0.0010	2.96 (1.56 to 6.35); p=0.0023

B

Predictor	**eGFR** (95% CI); p value	
	Univariate	Multivariable[a]
Proteinuria, log mg/g Cr	−14.78 (−24.63 to −4.93); p=0.0036	−8.11 (−17.40 to 1.17); ns (p=0.09)
Albuminuria, log mg/g Cr	−11.04 (−19.83 to −2.24); p=0.014	−6.10 (−14.29 to 2.09); ns (p=0.14)
Plasmin(ogen)uria, log μg/g Cr	−13.82 (−19.76 to −7.88); p<0.0001	−9.74 (−15.36 to −4.122); p=0.0008

[a]Variables selected for adjustment by a priori specification; included age, gender, race/ethnicity, use of drugs of time of biopsy: ACEi/ARB, diuretics, and eGFR (for edema model)