A novel assay provides sensitive measurement of physiologically relevant changes in albumin permeability in isolated human and rodent glomeruli

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Increased urinary albumin excretion is a key feature of glomerular disease but has limitations as a measure of glomerular permeability. Here we describe a novel assay to measure the apparent albumin permeability of single capillaries in glomeruli, isolated from perfused kidneys cleared of red blood cells. The rate of decline of the albumin concentration within the capillary lumen was quantified using confocal microscopy and used to calculate apparent permeability. The assay was extensively validated and provided robust, reproducible estimates of glomerular albumin permeability. These values were comparable with previous *in vivo* data, showing this assay could be applied to human as well as rodent glomeruli. To confirm this, we showed that targeted endothelial glycocalyx disruption resulted in increased glomerular albumin permeability in mice. Furthermore, incubation with plasma from patients with post-transplant recurrence of nephrotic syndrome increased albumin permeability in rat glomeruli compared to remission plasma. Finally, in glomeruli isolated from rats with early diabetes there was a significant increase in albumin permeability and loss of endothelial glycocalyx, both of which were ameliorated by angiopoietin-1. Thus, a glomerular permeability assay, producing physiologically relevant values with sufficient sensitivity to measure changes in glomerular permeability and independent of tubular function, was developed and validated. This assay significantly advances the ability to study biology and disease in rodent and human glomeruli.

Kidney International (2018) **93,** 1086–1097; https://doi.org/10.1016/j.kint.2017.12.003

KEYWORDS: albuminuria; angiopoietin-1; diabetes; endothelial glycocalyx; glomerular permeability; steroid-resistant nephrotic syndrome plasma

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Received 31 July 2017; revised 5 December 2017; accepted 13 December 2017; published online 9 February 2018

The ability to measure glomerular permeability is essential in the detection and assessment of glomerular disease. Albuminuria is the standard measure; however, it does not accurately reflect glomerular permeability. This is due to confounding changes in local hemodynamics, as well as tubular reabsorption of filtered albumin.[1] For experimental studies of human glomerular permeability, measurements of albuminuria are not possible. Therefore, the development of a reliable, physiologically relevant assay to directly measure glomerular permeability using isolated glomeruli would be very useful and independent of confounding variables. We have previously developed a reliable assay of water permeability in isolated glomeruli[2] and here we describe the development of an albumin permeability assay. In 1992, Savin *et al.* modified an isolated glomerular oncometric assay, originally developed to assess hydraulic conductivity,[3] to measure glomerular reflection coefficients[4] and further modified the assay in 2015 for higher thoughput.[5] However, this assay is limited to measuring relative changes in permeability. Daniels *et al.* developed an *ex vivo* method to quantify the apparent diffusion of fluorophores across individual glomerular capillaries in cross-section,[6] yet permeability values were significantly higher than values previously measured *in vivo*.[7] Using similar principles we developed an assay to measure apparent, diffusive glomerular albumin permeability in isolated glomeruli deriving physiologically relevant values.

We aimed to develop and characterize an assay that could sensitively measure glomerular albumin permeability and demonstrate its utility under different pathophysiological conditions, in different species. The pathophysiological variables tested were: (i) disruption of endothelial glycocalyx,[8–10] (ii) exposure to plasma from patients with recurrence of nephotic syndrome posttransplant,[11,12] (iii) early diabetes,[13,14] and (iv) rescue of glomerular permeability in diabetes using angiopoietin-1.[15,16]

RESULTS

Set-up and characterization of a novel glomerular albumin permeability assay

The abdominal aorta of rats and mice were cannulated to perfuse both kidneys (Figure 1a). Kidneys were perfused first with mammalian 4% Ringer bovine serum albumin (BSA) to flush out red blood cells (which interfere with analysis). They were then perfused with octadecyl rhodamine B chloride (R18, a lipophilic membrane stain) in 4% Ringer BSA, to label cell membranes red in order to distinguish the glomerular capillary walls. This was followed by perfusion with Alexa Fluor 488 (AF488; Thermo Fisher Scientific, Waltham, MA) labeled 4% BSA. Perfused glomeruli were isolated by sieving, then incubated in AF488-BSA with or without treatments. A single glomerulus was then placed in an adapted petri dish (Figure 1b) on a confocal microscope in AF488-BSA. This was trapped to micro-occlude the afferent and efferent arterioles using a glass micropipette, thereby sealing the glomerulus so that any movement of molecules could only occur across the capillary walls by diffusion. Regions of interest (ROI) were chosen within circular capillary loops (ROI1) and external to the glomerulus (ROI2, Figure 1ci). The 4% AF488-BSA was exchanged for iso-oncotic unlabeled 4% BSA, and the initial change in fluorescence intensity was measured over time in both ROI (Figure 1cii). The fluorescence intensity and concentration of AF488-BSA were linearly related (Figure 1d), the fluorescence intensity of AF488 was not impacted by time (Figure 1e), and AF488 free dye at 0.07% or below had no impact on fluorescence intensity (Figure 1f). The decline in fluorescence intensity in ROI1 was measured over 1 minute, which was the minimum length of time necessary to give sufficient data points to represent the initial decline (Figure 1g).

To demonstrate that macromolecules were effectively restricted by glomeruli in this assay, 2 different molecular weight dextrans conjugated with fluorescein isothiocyanate (FITC) were used. There was a linear relationship between low molecular weight (LMW) and high molecular weight

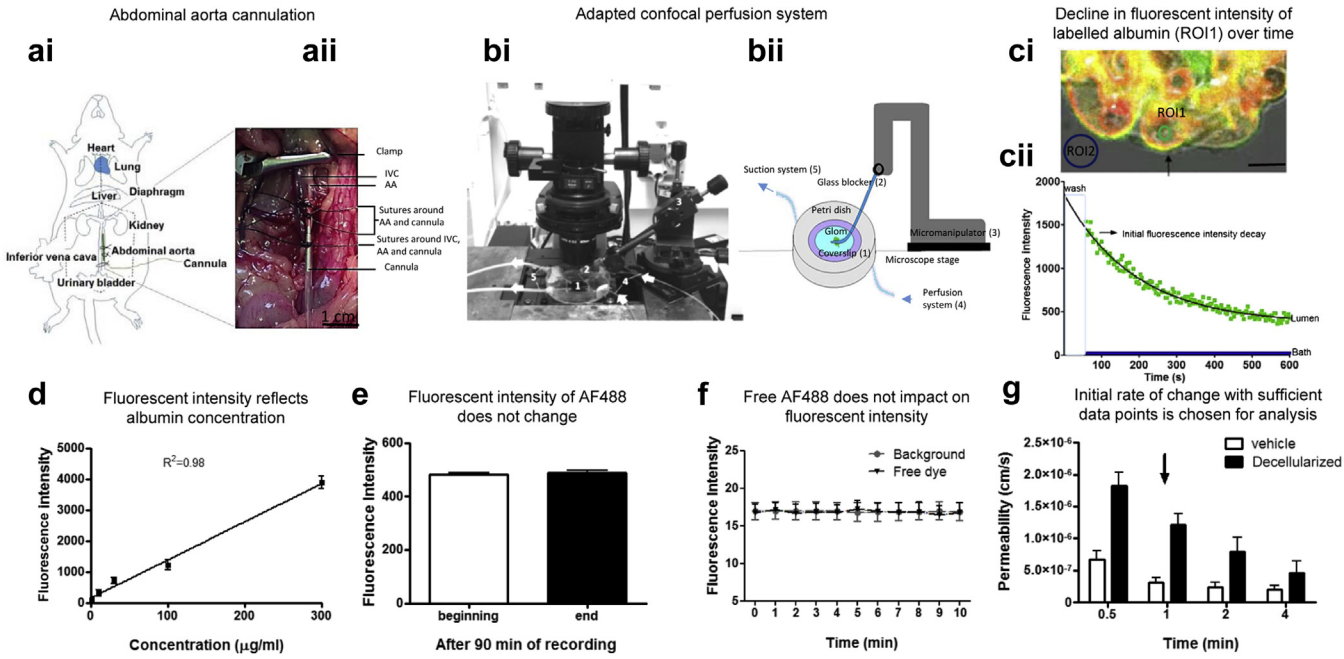

Figure 1 | Glomerular albumin permeability assay setup and characterization. (**a**) A midline laparotomy was used to expose the abdominal aorta, which was cannulated with a blunted needle, as detailed in (**i**) and (**ii**). AA, abdominal aorta; IVC, inferior vena cava. (**bi**) The photograph shows an inverted confocal microscope equipped with a modified Petri dish consisting of a central hole with a glass coverslip (0.085-mm thickness) attached on the top and connected to a perfusion and a suction system. Glomeruli were trapped on the (1) glass coverslip by a (2) glass restrainer positioned using a (3) micromanipulator. Ringer bovine serum albumin (BSA) was pumped into the Petri dish though a (4) perfusion system and simultaneously removed though a (5) suction system to wash the glomeruli of surrounding Alexa Fluor 488 (AF488) BSA (20–60 seconds). Arrows indicate the direction of flow. A schematic is shown to reflect the setup showing a glomerulus trapped by a micromanipulator connected to a restraining glass pipette. (**bii**) Numbers relate to those shown in (**bi**). (**ci**) An image of peripheral capillary loops of a trapped glomerulus is shown. The capillary wall is labeled red with R18, and AF488-BSA fills the lumen (labeled yellow where there is colocalization). The arrow indicates a spherical capillary, chosen for analysis. Region of interest (ROI) 1 and ROI2 are shown in the capillary lumen and bath, respectively (bar = 5 μM). (**cii**) The decline in fluorescence intensity of labeled albumin over time is plotted. The green boxes represent the flux of movement of fluorescently labeled albumin molecules from the lumen to the bath (ROI1), and the blue line represents the fluorescence intensity of the bath after the wash (ROI2). The decline in fluorescence intensity follows a single exponential decay (green boxes, $r^2 = 0.97$). (**d**) Mean fluorescence intensity was plotted against AF488-BSA concentrations. (**e**) The initial fluorescence intensity values were compared with the FI values at the end of the experiment (after 90 minutes of laser exposure; paired *t* test, $P = 0.20$). Free dye fluorescence intensity is plotted against time and is not different than background. (**f**) Permeability between decellularized and normal (vehicle-treated) glomeruli were analyzed for different periods of time following the washout period. (**g**) The period chosen for analysis after the washout period was 1 minute (arrow) because this had a significant number of frames to analyze (30 seconds was too few) and differentiated well between treatment groups. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.