Reply to “Letter to the editor: ‘Quantifying albumin permeability with multiphoton microscopy: why the difference?’”

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REPLY: Our recent study addressing the glomerular sieving coefficient for albumin (GSCA) and its modulation by angiotensin II resparked the controversy regarding the extent of glomerular albumin permeability (11, 23). Using multiphoton microscopy, we determined in rats that the permeability of the normal glomerular filtration barrier to albumin is ultra-low. Thus the GSCA was in the range of 0.0005 and increased substantially during the infusion of angiotensin II (23). This GSCA value is similar to those reported previously by two independent laboratories using the same method (12, 16, 17, 20, 25) but differs markedly from what has been reported by the group of Russo, Sandoval, Comper, Molitoris, and coworkers (19, 21, 22). These authors reported that the normal glomerul滤 albumin at much higher levels (19, 21, 22). These large amounts of filtered albumin were suggested to be subsequently reabsorbed by the proximal tubule and eventually released into the circulation as an intact protein via transcytosis. The GSCA originally reported by this group was 0.034 (~2 orders of magnitude higher than our results); in follow-up studies, they reported a value in the range of 0.008–0.035, depending on both the rat strain and the fasting state of the animals (22).

What might be the reason for this apparent discrepancy? The determination of fluorescence intensities over a range of several orders of magnitude, such as for labeled albumin in the glomerular capillary loop vs. Bowman’s space, is demanding. Because the fluorescence intensities of albumin in Bowman’s space are low, the adjustment of the background fluorescence by changing the offset can be argued to potentially lead to compromised detection sensitivity (21). We agree that excessively large negative offsets may be an issue when approaching the lower limit of detection (2). The offset used in our study reduced the background to low levels but was within a linear correlation of background intensity/offset. Furthermore, in contrast to the results of Sandoval and Molitoris for a large offset (offset 45 in Fig. 1A) (21a), the intensities of labeled albumin measured in our study were within the linear range of fluorescence intensities vs. concentration. We therefore consider it unlikely that our data suffer from a considerable lack of sensitivity, which would result in erroneously low GSCA values. Furthermore, background intensities were determined before the infusion of the labeled albumin and were subtracted from all postinfusion measurements (23).

The underestimation of the fluorescence intensity within the glomerular capillaries (translating into erroneously high GSCA values) due to the absorbance by red blood cells has been suggested as another potential pitfall (16, 17), but this possibility was questioned in a later study (22).

Based on our own experiments, we believe that out-of-focus fluorescence (in particular when using external, nondescanned detectors) may lead to the substantial overestimation of the fluorescence intensities of labeled albumin within Bowman’s space when measurements are made in close proximity to glomerular capillary loops above or below the focus plane (25). Thus, with the use of external detectors, the fluorescence intensity of the Alexa Fluor 594 BSA conjugate was constant at a distance of ≥5 μm above or below the capillaries (approximately half the diameter of the capillary), but was increased up to 30-fold when the intensities were determined adjacent to the outer margin of the capillary wall (considering podocytes to be part of the capillary wall structure). This issue is illustrated in Fig. 1. The measurement of this apparent out-of-focus fluorescence will translate into erroneously high GSCA values. Although multiphoton microscopy in principle is focal by excitation, the extension of the effective two-photon excitation volume (along the z-axis) is not infinitely small (29). Furthermore, the concentration of the fluorescent albumin in the capillary loops is more than three orders of magnitude higher compared with those measured in Bowman’s space, suggesting that even rare excitation events may lead to the collection of considerable fluorescence intensities. This out-of-focus fluorescence may be particularly relevant when imaging is performed close to the surface of the organ (26), which is the preferred set-up to obtain high image quality with low laser power (22). As a consequence, the positioning of the region of interest may considerably influence the intensities determined in Bowman’s space and subsequently the GSCA. Therefore, collecting z-stacks of the glomerulus before measuring the intensity in Bowman’s space appears advisable. This step should be performed before every experiment to account for subtle movements of the kidney. With the knowledge of the 3D-environment of the region of interest within Bowman’s space, close proximity to capillary loops can be avoided when choosing a region for intensity measurements.

In addition to the technical aspects of intravital multiphoton microscopy, the physiological condition of the animal appears to be crucial and may considerably influence the GSCA. Because only limited data on variables such as blood pressure (for example, only an average value for the mean arterial pressure for the entire imaging session is given in Ref. 22) are shown in the studies that generated high GSCA values, reconciling whether these variables may have influenced the determination of the GSCA is difficult. Our results suggest that insufficiently controlled physiological conditions associated with hypothermia, volume loss, low arterial blood pressure, and low renal blood flow (the latter may be a problem depending on the positioning of the animal), which can all lead to an activation of the renin-angiotensin system (5), may markedly increase the GSCA by angiotensin II-dependent mechanisms (23). Different anesthesia protocols are another possible variable, although they have not been addressed systematically. For example,
different anesthetic agents were used within a single study, such as sodium pentobarbital in one strain of rats and inactin in another (22).

The way out: considering other methods. When a discussion about the adequacy of a specific method comes to a dead-end, it is often advisable to considering another, independent methodic approach. In fact, an evaluation of the GSCA was performed in multiple studies, long before any multiphoton microscopy was available. The findings of these studies, most of which were performed using micropuncture techniques, considerably contributed to the traditional view that the normal GSCA is very low. Thus early micropuncture studies suggested that the GSCA was ~0.0003 (15, 24). There are potential pitfalls with the micropuncture approach, such as possible contamination by extratubular proteins (which would result in an overestimation of the GSCA) or the absorption of albumin by the pipettes (which would result in an underestimation of the GSCA). The authors were aware of these issues, and highly sophisticated methods were developed to circumvent these problems. In the likely most elaborate study, Tojo and Endou (27) reported steady-state tubular albumin concentrations of 10 µg/ml in consecutive fluid collections done with double-pipettes, resulting in a GSCA of 0.0006. In the first of these collections, the albumin concentration, however, was higher (17 µg/ml), and the authors suggested that this difference might be due to contamination caused by the insertion of the micropuncture pipette. Regardless of the exact reason for these changes over time, the corresponding GSCA for the highest albumin concentration would then be 0.001 instead of the calculated 0.0006, which is still more than 30-fold lower than originally reported by Russo (19). Similar results were obtained by micropuncture to collect fluid samples directly from Bowman’s space (18). Feeding may increase the GSCA (22), but, contrary to what was stated in a recent review (8), a closer inspection of the methods sections of the cited micropuncture studies indicates that these experiments were usually not performed in fasted animals (27). Low GSCA values in the same range were also calculated by an indirect approach using an isolated, perfused kidney, in which proximal tubular function was reduced by cooling to 8°C (14).

Some additional considerations: the fundamental principles of glomerular physiology. A massive tubular (presumably proximal tubular) uptake and the transcytosis of filtered albumin would be a prerequisite for the consistency of the concept of a high albumin filtration level, given that urinary albumin excretion is normally minimal (≤30 mg/day in humans). In their letter, Sandoval and Molitoris (21a) argued that the hypothesis of a high albumin filtration level under normal conditions is supported by the finding of albuminuria in injury models of the proximal tubule, such as due to the expression of a diphtheria toxin receptor (10). At first glance, this hypothesis seems plausible, but the increase in albumin excretion in this situation was minor, and albuminuria correlated with the degree of glomerular sclerosis (10). In another study, Amsellem et al. (1) used mice with targeted deletions of proximal tubular cubilin, megalin, or both, to address their role in tubular albumin uptake. In contrast to wild-type proximal tubular cells, no albumin-containing vesicles were found in either cubilin- or megalin-deficient cells (1), suggesting that the bulk of proximal tubular albumin retrieval is cubilin/megalin dependent and that other receptors, such as the neonatal Fc receptor (FcRn), may account for the intracellular sorting of albumin rather than its cellular uptake (6, 8). Nevertheless, urinary albumin excretion was only increased sixfold in cubilin-deficient mice, similar to the results reported for megalin-deficient mice (3-fold) (3). These findings appear difficult to reconcile with the concept of a high albumin filtration level. Translated into the situation observed in humans, the albumin excretion of cubilin-deficient mice would correspond to a daily albumin excretion of 6 × 30 mg/day = 180 mg/day (assuming ≤30 mg/day being normal), which would not be considered to be massive albuminuria, particularly when taking into account that the daily filtration of albumin in humans according to the high-GSCA concept would be >200 g/day (7, 9). It should be noted in this context, that patients with Fanconi’s syndrome (generalized proximal tubular dysfunction of variable etiology) suffer from low-molecular weight proteinuria (which may include some degraded albumin if proximal tubular albumin degradation pathways are preserved) with little albuminuria (13, 28), whereas the excretion of the freely filtered glucose my increase up to 1,000-fold (4), suggesting again that the filtration of albumin in the normal kidney is minimal. According to the high GSCA concept, even subtle changes in proximal tubule function would be expected to cause massive alterations in albumin excretion, but this outcome does not always appear to be the case. There might be alternative pathways of intact
albumin retrieval (presumably downstream of the proximal tubule), but to date this possibility is mere speculation.

DISCLOSURES

No conflicts, of interest, financial or otherwise, are declared by the author.

AUTHOR CONTRIBUTIONS

H.C. drafted manuscript; H.C. edited and revised manuscript; H.C. approved final version of manuscript.

REFERENCES