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REPLY

Podocytes are key—although albumin never reaches the slit diaphragm

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We would like to thank Dr Comper for his correspondence on our Review (Renal albumin filtration: alternative models to the standard physical barriers. *Nat. Rev. Nephrol.* **9**, 266–277; 2013)[1] and the others in the *Nature Reviews Nephrology* collection, which raises two important issues that must be addressed (Albuminuria is controlled primarily by proximal tubules. *Nat. Rev. Nephrol.* 28 January 2014; doi:10.1038/nrneph.2013.58-c1).[2]

Firstly, Dr Comper states that "Nephrology students who read these articles would get the incorrect impression that the glomerulus and/or podocytes are central to development of albuminuria." Contrary to what Comper proposes, the majority of researchers agree that the primary filtrate is virtually free of albumin; that is, the glomerular sieving coefficient (GSC) of albumin is extremely low (close to 0.001).[3,4] Several lines of evidence support this position.[1,3] For example, micropuncture studies in the rat kidney have demonstrated very low albumin concentrations in the primary filtrate. In cooled or fixed isolated perfused kidney, cubilin/megalin knockout mice or human patients with Fanconi syndrome, only very little protein is excreted in the urine (<1 g albumin per day in humans). Additionally, filtered protein has been observed in Bowman's space in histological sections only in proteinuric rodents, but not in normal controls. Other mammalian filtration systems also show a very low albumin sieving coefficient—for example, the choroid plexus in humans has a sieving coefficient of 0.003–0.0008. Finally, mutation of a podocyte-specific gene (*NPHS2*, which encodes podocin) is sufficient to result in nephrotic-range proteinuria.

Why is the podocyte essential for glomerular integrity? The glomerular filtration barrier challenges us with some formidable 'riddles' and controversy abounds as to how it functions. One of these riddles is the observation that most of the albumin in mammalian kidneys never actually reaches the podocyte slit diaphragm as visualized by rapid *in situ* fixation techniques and immunoelectron microscopy.[5–7] Albumin (as well as other tracer molecules, such as ferritin) is repelled at the level of the endothelium, which argues in favour of a low GSC and—on first look—seemingly supports Comper's hypothesis that the podocyte might not have a role in proteinuria.

However, the electrokinetic model of glomerular filtration[1] can provide a relatively simple mechanistic explanation for many of the riddles of the glomerular filter. In this regard, the filter can be viewed not only as a passive porous barrier (as in the pore model) but a barrier with electrical effects. Specifically, the glomerular filter produces streaming potentials, which are generated whenever an ionic fluid passes through an electrically charged barrier. Thus, filtration generates a homogeneous electrical field across the glomerular filtration barrier (on the order of approximately 0.1 mV/300 nm, or a few hundred volts per metre). This electrical field is sufficient to repel negatively charged plasma proteins from entering the filtration barrier. Accordingly, the electrokinetic model predicts that any insult that impairs homogeneous filtration will interfere with the generation of a homogeneous electrical field and result in proteinuria.[1]

Injury to podocytes usually leads to a uniform response: effacement of foot processes. As a consequence, larger parts of the glomerular filter surface will block filtration because they are covered by flattened podocytes. In turn, the electrical field decays in these areas and plasma proteins are only incompletely repelled and pass into the primary filtrate—resulting in proteinuria. Interestingly, the electrokinetic model also provides explanations for other riddles: for example, why the filter becomes more permeable when filtration pressure is decreased below the autoregulatory range (as in orthostatic proteinuria), and why the filter never 'clogs'.[1]

Next, Comper states that out-of-focus fluorescence has no physical basis in two-photon microscopy and cannot explain high albumin GSC values. The two-photon microscope is a powerful new tool that can be used to measure the intensity of fluorescently labelled albumin in Bowman's space and glomerular blood plasma *in vivo*, thereby enabling direct measurement of albumin GSC in laboratory animals. The fluorescence signal recorded from Bowman's space is extremely low and close to the background.[4] When imaging with the two-photon microscope in a medium that does not scatter light (for example, a clear aqueous solution of fluorescein in a cuvette), the fluorescence generated is confined to the small focal region of the incident beam. In biological tissues, however, excitation and emission light is highly scattered, resulting in low intensity at the focal region and fluorescence generated away from the focus (hence, out-of-focus fluorescence). Much of this fluorescence is generated close to the surface of the tissue and it increases with the depth of imaging.[8,9] Furthermore, out-of-focus fluorescence is more readily detected if external photodetectors are used than if internal photodetectors are used. Even when wide open, a confocal pinhole used in internal photodetector mode blocks most of the out-of-focus fluorescence.[9]

The existence of out-of-focus fluorescence as a source of error in GSC measurements was clearly demonstrated in a study in which the GSCs of two very large molecules, thyroglobulin and dextran 2,000,000, were measured in Munich–Wistar rats.[4] To remove low molecular weight fluorescent contaminants, the molecules were filtered in centrifuge tubes with a 100 kDa cut-off value prior to intravenous injection. The GSCs of these molecules were

zero when internal detectors were used, but greater than zero when external detectors were used, a finding best explained by detection of out-of-focus fluorescence in the external detector mode. In the same study, an albumin GSC of 0.002–0.004 was determined using internal detectors. External detectors were used in the two-photon study from Comper's group[10] and, accordingly, the albumin fluorescence measurements in Bowman's space were likely contaminated by out-of-focus fluorescence. This, and other factors, explains their high value of 0.034 for albumin GSC.[4]

Recently, three two-photon microscopy studies[11–13] reported very low albumin GSCs (approximately 0.0006). Sandoval *et al.*[14] criticized these studies because large negative voltage offsets were applied to external photodetector amplifiers, which can result in inaccurate, low values. It would have been helpful if the offset had been adjusted only to the point where very few pixels in the background image reported zero, and if out-of-focus fluorescence detection had been dampened using internal detectors or corrected for by measuring the sieving of large nonfilterable molecules.[4]

In summary, the electrokinetic model can reconcile many seemingly controversial experimental findings on the glomerular filtration barrier. Two-photon microscopy is a potentially powerful new tool to study glomerular filtration, but we might still be within a learning curve regarding its limitations.

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Competing interests
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