Chapter 9

Evaluation of the Glomerular Filtration Barrier by Electron Microscopy

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Additional information is available at the end of the chapter

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Abstract

The plasma filtration and formation of the urine is a very complex process necessary for the elimination of metabolites, toxins, and excessive water and electrolytes from the body. The initial process of urine formations is done by the glomerular filtration barrier inside the glomeruli. This specialized barrier consists of three layers, fenestrated endothelium, basement membrane, and podocytes, which ensure that water and small molecules pass through while cells and large molecules are retained. The glomerular filtration barrier is found with abnormal morphology in several diseases and is associated with renal malfunction; thus, it is interesting to study these structures in different experimental and clinical conditions. The normal glomerular barrier and its alterations in some conditions (hypertension, diabetes, and fetal programming) are discussed in this chapter. Furthermore, some methods for studying the glomerular filtration barrier by electron microscopy, both by qualitative and quantitative methods, are present.

Keywords: Electron microscopy, glomerulus, filtration barrier, morphometry

1. Introduction

The human kidney is responsible for many functions, such as filtration of the plasma, blood pressure control, and hormonal production among others. Kidneys are bean-shaped organs located in the retroperitoneal space, irrigated by the renal artery, and covered by its fibrous capsule. Renal parenchyma can be distinguished into cortex and medullar regions, with different anatomic–histological characteristics. Inside a human kidney, there are about one million nephrons, which are composed of tubules and renal corpuscles. The corpuscles contain several tortuous arterioles covered by podocytes. The set of the endothelial layer of these

arterioles, its basement membrane, and the podocytes correspond to the glomerular filtration barrier [1, 2].

The ultrafiltration of the plasma by this barrier and formation of the primary urine requires normal glomerular morphology. It is well documented that the podocytes are important to guarantee the selectivity during the filtration process, preventing macromolecules that pass through the slit diaphragm formed between its foot processes. Many studies suggest that alterations on these cells or on the vessels, as those during hypertension, diabetes, or lupus, can reduce the oxygen flux, leading to hypoxia stage, being responsible for podocyte alteration and death [3, 4].

The glomerular filtration barrier morphology has been extensively studied in several experimental and clinical conditions. For this purpose, electron microscopy (both transmission and scanning) suits perfectly for observing the fine structure of this barrier, indicating with accuracy morphological alterations when present [5, 6].

Thus, in this chapter, we aimed (a) to describe the elements of the glomerular filtration barrier, (b) to present scientific examples of how the glomerular filtration barrier elements are related to different clinical and experimental conditions, and (c) to show how the elements of the glomerular filtration barrier can be analyzed by scanning and transmission electron microscopy with qualitative and quantitative methods.

2. Main body

2.1. The normal glomerular filtration barrier

The glomerulus is a highly irrigated structure that performs selective filtration of the plasma. Inside the Bowman's capsule, several tortuous arterioles receive the blood and filtrate it forming the primary urine, which then passes to the proximal tubule. The glomerular capillaries are lined by a fenestrated endothelium, covered externally by specialized cells, called podocytes. Between the cell layers, there is a basement membrane, which also has an important filtering function. Together, the endothelial cells, the basement membrane, and the podocytes form the glomerular filtration barrier [1, 3].

The glomerular filtration barrier is highly permeable to water and small molecules. Moreover, it is slightly permeable to macromolecules and acts as a physical and electrical barrier for the filtration process. These characteristics are dependent of the cellular structures, and its function is influenced by factors such as molecular weight and electric charge [2]. Moreover, changes in the cell junctions of the glomerular barrier prejudice the glomerular function [7].

Internally, the glomerular filtration barrier is constituted by the glomerular fenestrated endothelium. These endothelial cells have a glycocalyx over its luminal surface, which form a highly negatively charged coating [2]. Thus, glycocalyx covers the endothelium and promotes a first selection of molecules passing through the barrier by electric charge. The endothelium coating the glomerular capillaries is very thin and has several fenestrae with 70–90 nm of diameter. These pores can filtrate only large molecules and blood cells [1].

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The endothelial cells are supported by a basement membrane (about 100–150 nm), which is the only continuous layer of the glomerular filtration barrier. As this membrane is thought to be the fusion of the endothelial and epithelial basement membranes, two laminae lucida (interna and externa) and, between them, a lamina dense are found [1]. The basement membrane is composed of a complex network of glycosaminoglycans and fibrous proteins (laminin and collagen type IV), which are continuously produced and deposited by podocytes and mesangial cells [8]. These proteins adhere to the cell membranes by surface receptors and form the glomerular filtration barrier [5].

Podocytes are specialized epithelial cells found in the external layer of the glomerular filtration barrier and exhibit several long cellular processes, which bears various secondary processes, named as foot processes. The foot processes involve capillaries by interdigitations, and small gaps are left in between. These small gaps measure about 20–30 nm and is named as slit diaphragm being responsible for passage of small molecules, whereas larger ones are retained [6]. Further, the slit diaphragm is filled with nephrin and podocin, the transmembrane proteins that are also important for the correct function of glomerular filtration barrier. Experimental studies have shown that mutations in expression of these proteins can alter the filtration barrier and probably is the cause of nephrotic syndrome [9].

In addition to adhesion receptors from integrin family, there are also proteoglycan transmembrane receptors such as those of syndecan family. Also, the junction of podocytes membrane is seen by electron microscopy, showing zipper-like structure where binding proteins form cell junctions and small pores, as previously mentioned on filtration slit [6]. Thus, it is important to consider all glomerular ultrastructure in the filtration process and renal function. Some diseases provoke changes in glomerular ultrastructure, which promote irregular filtration rates, proteinuria, and even kidney failure, further discussed here. Electron microscopy and correlate techniques allow better to understand these pathological changes and become an effective and important method in the study of renal function.

2.2. The altered glomerular filtration barrier

Literature describes well several diseases that affect the glomerular filtration barrier. Some conditions such as diabetes mellitus, hypertension, and maternal nutritional changes during critical periods of development, known as fetal programming, are well recognized as important risk factors for developing chronic kidney disease.

Diabetes mellitus is one of the most chronic diseases emerging on twenty-first century, in which hyperglycemia is a major indicator, generating microvascular damage such as retinopathy, neuropathy, and kidney disease [4]. Diabetic nephropathy is a chronic progressive disease that affects 20–40% of patients with diabetes mellitus [10]. Histopathologically, this disease in humans courses with the thickening of glomerular and tubular glomerular basement membrane, podocytopenia, mesangial expansion, glomerular, and arteriolar hyalinosis. The earliest clinical manifestation of diabetic nephropathy is microalbuminuria, a strong predictor of renal and cardiovascular disease in patients with type 1 and type 2 diabetes mellitus [11, 12]. These modifications contribute to the abnormal stimulation of resident kidney cells, which increases the production of TGF-β1 and causes collagen (types I, IV, V, and VI), fibronectin,

and laminin depositions in the extracellular matrix of the glomerulus. Thus, this structural disorganization of the glomerular slit diaphragm enhances renal damage and chronic kidney disease progression [13, 14].

Likewise, hypertension is directly related to renal failure [15]. Hypertensive nephropathy, a consequence of chronic increase of blood pressure, is secondary to diabetic nephropathy in terms of diagnosis and is considered the last stage of renal disease. Some complications are associated with hypertensive nephropathy such as glomerular damage resulting in impaired renal function [16]. Hypertension causes an increase of numerous local factors, such as angiotensin II, which may contribute to the development of renal fibrosis. It is reported that angiotensin II stimulates the gene expression of TGF-β1 and the release of this protein. The presence of TGF-β1 activates the conversion of fibroblasts into myofibroblasts, producing large amounts of extracellular matrix components, and induces renal fibrosis [17].

Moreover, angiotensin II regulates the number and integrity of podocyte. In high quantities, it promotes the disintegration and breakup of these highly specialized cells and causes glomerular endothelial cells hypertrophy since it raises intraglomerular pressure, preferably affecting the afferent glomerular arterioles [18]. The increase of blood volume is also capable to reduce the levels of podocin (abundant protein in the podocyte body) and nephrin (a structural component of the slit diaphragm) in the glomerulus, which enhances the glomerular podocyte injury and albuminuria [19].

Recently, experimental and epidemiological studies report that several metabolic disorders manifested in adulthood have their roots dating embryonic periods [20, 21]. Protein or energy restriction during pregnancy induces low birth weight and, consequently, the injury of nephrogenesis, increasing the incidence of chronic kidney disease in adulthood [22]. As previously mentioned, the glomerulus (the most important filtering apparatus in the body) is a highly specialized structure formed by four types of cells: mesangial, endothelial, visceral (podocytes), and parietal epithelial cells. Accordingly, the intrauterine growth restriction induced by maternal protein restriction may cause morphological and functional changes in the glomerulus, thereby decreasing the filtration barrier efficiency with consequent glomerulosclerosis [23].

Just as occurs in diabetic nephropathy, low birth weight promotes glomerulosclerosis [24], expansion of mesangial matrix, hyalinosis, and podocytopenia, which compromises glomerular filtration and facilitates progressive kidney dysfunction [25]. The loss of podocytes may represent the starting point for an irreversible glomerular injury, characterized by proteinuria and glomerular scarring. Transmission electron microscopy analysis revealed that the offspring from maternal low protein diets at 26 weeks of age and at 16 weeks old and presents the effacement of pedicels, the absence of the slit diaphragm, and an increase of glomerular basement membrane thickness, denoting a reduced of barrier efficiency filtration [24, 26].

Another metabolic programming model related to the occurrence of chronic kidney disease in adult life is maternal vitamin D restriction. This vitamin is essential for the development of the nervous system, immune function, skeletal formation, and fetal kidney [27, 28]. Studies in rats showed that vitamin D restriction during critical periods of development has increased the

number of glomerulus, reduced the size of renal corpuscles, and delayed glomerular maturation, further lower expression of WT1 and podocin in adult offspring [28, 29]. These structural and functional adaptations in the glomerulus may progress to chronic kidney disease

2.3. Glomerular filtration barrier evaluation

2.3.1. Sample preparation

A very good sample preparation for EM must follow careful cares because small mistakes will be big ones when analyzed by EM. Thus, we can use either old or new approaches when we intend to study not only the glomerulus but also any biological sample.

However, the glomerulus is particularly difficult due to different osmolarities found in each segment of the nephron. Cryofixation, in general, needs much smaller samples sizes than conventional chemical fixation. A freeze-depth of about 10–15 μm is achieved; one exception is high-pressure freezing (HPF). Nowadays, there are new techniques and good, although expensive, equipments commercially available such as high-pressure freezing (HPF), slam freezing, cryoultramicrotomy, freeze-fracture, freeze-etching, and so on. New scanning electron microscopies with high resolution and extreme high resolution that appeared recently provided beautiful and illustrative aspects of all different cell types. Concerning transmission EM, new methodologies allow 3-D reconstruction such as tomography of thick or semithick sections, serial sections, or high-voltage TEMs. In addition, spectacular images were recently obtained with new Helium ion (proton) microscopes.

When sample preparation for the evaluation of the glomerular filtration barrier by electron microscopy follows the standards of routine biological sample preparations, the person should manipulate the tissue with the animal alive, under anesthesia, for obtaining a small fragment with a size of 0.5 mm or even less and immediately immerse it in the fixative. Alternatively, perfusion gives very good results.

When studying biopsy samples of human kidney or whenever the perfusion is not possible or recommendable, fixation by immersion is an adequate option. For this, a small fragment of the renal cortex is collected, washed in buffered solution, and immediately immersed in the fixation solution [30]. This fragment should be collected from the outer part of the renal cortex, avoiding juxtamedullary region where glomeruli are sparser [1]. As routine preparation, fixation of the kidney is done with glutaraldehyde alone or in combination with freshly prepared formaldehyde from paraformaldehyde powder in warm water. The formaldehyde molecule is a very small aldehyde and has the advantage to penetrate rapidly into tissue, although it is not a strong linker for proteins. Thus, a good procedure is its combination with glutaraldehyde since it penetrates more slowly than formaldehyde and provokes cross-links with proteins. However, glutaraldehyde is a slow fixative, and it takes some seconds or even some minutes to cause death to cells and tissues. The small fragments are fixed in 2.5% glutaraldehyde or 4% PF with 2.5% glutaraldehyde, diluted in 0.1 M cacodylate buffer, pH 7.2, overnight at room temperature. Most important, buffered solutions should be used for better preservation of the cellular aspects of the glomerular filtration barrier [30, 31]. In the next day, samples are post-fixed in 0.1 M cacodylate buffer containing 1% OsO_4 and 0.8% potassium

ferricyanide for 1 h. It is important to point out that potassium ferricyanide is an important approach to better visualize the trilaminar structure of cell membranes. Then samples are dehydrated in graded series of acetone or alcohol. Ultrathin sections obtained after epoxy resin embedding are stained with uranyl acetate and lead citrate and observed in transmission electron microscope.

When studying samples collected from large animal models (i.e., pig, sheep, dog, etc.), the whole kidney can be adequately fixed by perfusion through the renal artery. When studying small species of animals (i.e., rats, mouse, etc.), the whole animal can be perfused through the left ventricle or aorta. In both cases, the researcher may take into account that some fixatives could hamper some other analysis, as for example, perfusing the kidney with glutaraldehyde may prejudice immune-labeling analysis, unless used in very low concentrations such as 0.5% glutaraldehyde or less [31]. We recommend fixation by perfusion in a mixture of glutaraldehyde and freshly prepared formaldehyde because the latter is a fast fixative allowing not only good preservation but also a quick cell death. Whenever using perfusion fixation, the osmolarity of the fixative solution should be taken into account. Preferentially, the osmolarity of the solution for kidney perfusion fixation should be of 420 mOs [32]. After organ perfusion, a small fragment of the renal cortex should be collected and immersed in the fixative solution for postfixation and dehydration as describe by immersion above. It is important to remember removing the renal capsule whenever it is present, allowing the fixative penetrate into the tissue of interest. Also, cryofixation can be an option for sample fixation for glomerular filtration barrier evaluation. One advantage of high-pressure freezing technique is that larger fragments with edges as large as 1 mm can be used, being a good choice as a cryofixation method [31].

As the object of interest is present in the glomerulus, we may take into account that our samples should have at least one glomerulus for examination. The glomerulus of rats has an average area (in its maximum cross section) of 0.02 mm^2 and occupies around 7.5% of the surface area on renal cortex [33]. Thus, for improving the chance of having a glomerulus on our sample, the examined surface area of the sample should be of at least 0.5 mm^2 (0.02 × 200 / 7.5). Based on this, the cortical fragment collected for glomerular filtration barrier analysis with electron microscopy should be about 1 × 0.5 × 0.5 mm in size. Furthermore, as there is no guaranty of having glomeruli in the sample, several fragments must be collected, processed, stored, or fixed.

It is important to point out one step that should not be overtaken: obtaining and observation of semithin sections before the ultrathin sections. Semithin sections are important because it is necessary (a) to confirm that there is a glomerulus in the cutting surface of the sample, (b) to determine whether the pyramid in the block face is correct in size and shape or should centered over a glomerulus, and (c) to observe the hole cutting surface under low magnification and obtain a copy in light microscopy that will be useful for searching glomeruli when this sample is observed with electron microscopy. It is very often that when the semithin sections are observed, the block is taken away as no glomerulus was found on its surface. This reinforces the importance of collecting several fragments of each animal/patient for electron microscopy analysis.Alternatively, electron tomography can be used. For this, thick sections (>200 μm) and

intermediate (200–300 kV) or high-voltage (750–1000 kV) EM equipped with a eucentric tilting stage are used. Colloidal gold particles (10 nm) are deposited on surface of the grids, to be used as fiducial markers for alignment of the tilted views. Single-axis tilt series from –60° to 60° images are collected in 1° increments using TEM coupled to a 4k × 4k CCD camera. About 400 images are obtained and three dimension reconstruction and data analyses are performed using proper software package as recommended by the EM company.

Sample preparation for SEM requires the same care as for TEM. Aldehyde fixation is adequate, but in order to improve lipid stability, contrast, and electron beam scanning, osmium tetroxide is also used. Sample is dehydrated with the same agents for TEM; however, we prefer to use alcohol starting from 7.5% to three times in 100% to avoid problems of drying artifacts. Next, tissue will critical point dried and coated with a very thin layer of gold or other conductive metal, producing beautiful images but without high resolution.

2.3.2. Helium ion microscopy

Recently, the group of Rice et al. [34] used helium ion microscopy (HIM) to analyze the rodent glomeruli and obtained wonderful images of uncoated samples by SEM. The authors used transcardial perfusion with aldehyde fixatives, vibratome sectioning, gradual dehydration in methanol series, and a very careful critical point drying. There is no need to apply conductive coatings to the samples prior to imaging, and thus sample surface information is preserved. Even at low magnification, the high quality and depth of field of HIM images is impressive. The images obtained with HIM are important and beautiful. The authors depicted some nanoprojections of the foot processes membrane (Figure 1). Also, podocytes and endothelial fenestrae within the filtration slit diaphragm were observed with much more detail (Figure 2). Nanoprotrusions originating from the processes are clearly seen projecting into urinary space. The future will provide much more resolution for biological samples.

2.3.3. Qualitative analysis of the elements of the glomerular filtration barrier

The glomerular filtration barrier can be easily evaluated by simple observation with electron microscopy. One aspect commonly evaluated is the loss of characteristic fenestration of the endothelial layer, which has been reported in some medical conditions previously described. By transmission electron microscopy, the endothelial cells are seen lying over the basement membrane and gaps of this layer, which correspond to fenestrae are fewer when compared to normal conditions [1].

The glomerular basement membrane is another part of the barrier easily evaluated with transmission electron microscopy. By routine examination, the membrane thickness can be qualitatively assessed. As the thickness of the membrane is of great importance for proper functioning of the filtration barrier, this is of interest in many studies. In addition, alterations found in basement membrane are highly associated with several diseases, as previously mentioned, reinforcing the importance of study this structure.

Finally, the podocytes, the third layer of the glomerular filtration barrier, are probably the most studied structure of this barrier. One important aspect of these cells is the interlacement formed

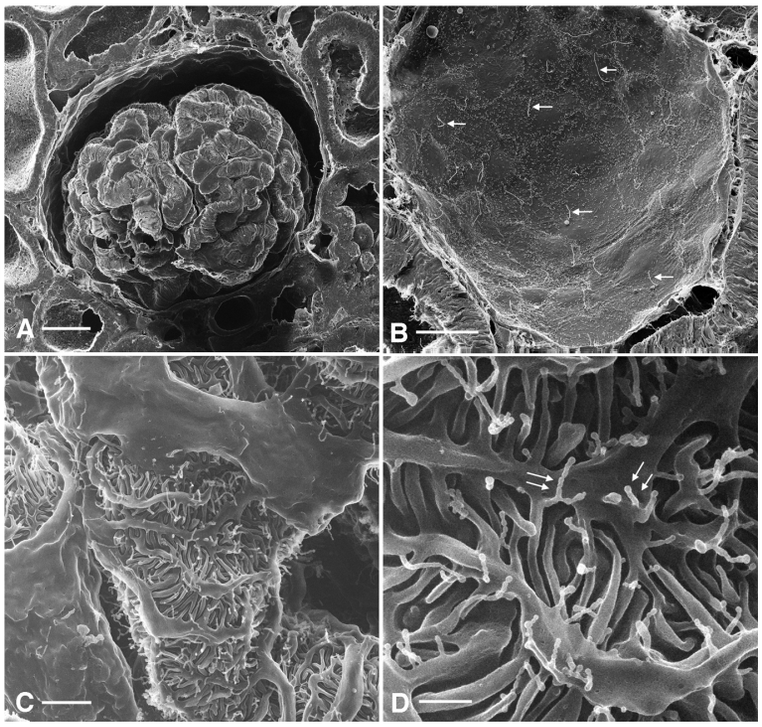

Figure 1. Microscopic images of glomerulus of rats as seen by scanning helium ion microscopy. In image A, we see the glomerulus in low magnification and several cut open tubules surrounding the glomerulus. Bar = 20 mm. In image B, the interior of a Bowman's capsule from which the glomerular tuft was removed is observed. Each parietal cell displays a single, long central cilium (arrows) that is very well preserved and visualized without heavy metal coating commonly used in scanning electron microscopy but unnecessary for ion microscopy. Bar = 10 mm. In image C, with intermediate magnification of the surface of a glomerular tuft, it is showed the complex interdigitations of podocytes and their foot processes. Bar = 2 mm. Finally, under higher magnification, it is possible to observe in image D that the podocyte processes are decorated by fine, thread-like protrusions (arrows). Bar = 0.5 mm. Image courtesy of Prof. Dennis Brown [34], available at doi:10.1371/journal.pone.0057051.g001.

by its foot processes, with a small slit diaphragm in between [35]. The alteration of the normal morphology of podocytes and foot processes is named effacement, which has been demonstrated in several conditions [36]. Podocyte effacement is characterized by loss of the normal interdigitations pattern of foot processes, leaving a thinner cell covering a large area outside the basement membrane. As consequence, morphological modifications as fewer and wider foot processes of podocytes are found and can be observed either with transmission or scanning electron microscopy. Among the advantages of observation with scanning electron microscopy are the following: (a) deep field, (b) possibility of analysis of larger areas, allowing the visualization of several podocytes and foot processes in the same sample, and (c) beautiful images of the glomeruli within Bowman's capsule, which are easy to understand and interpret, as shown in figures 3 and 4.

Qualitative differences found in glomerular filtration barrier from individuals submitted to any experimental or clinical condition are only possible when comparison is done with health organisms. However, only qualitative studies do not allow a deeper analysis and statistics and thus lacks scientific excellence. In addition, qualitative observation leads to problems of the

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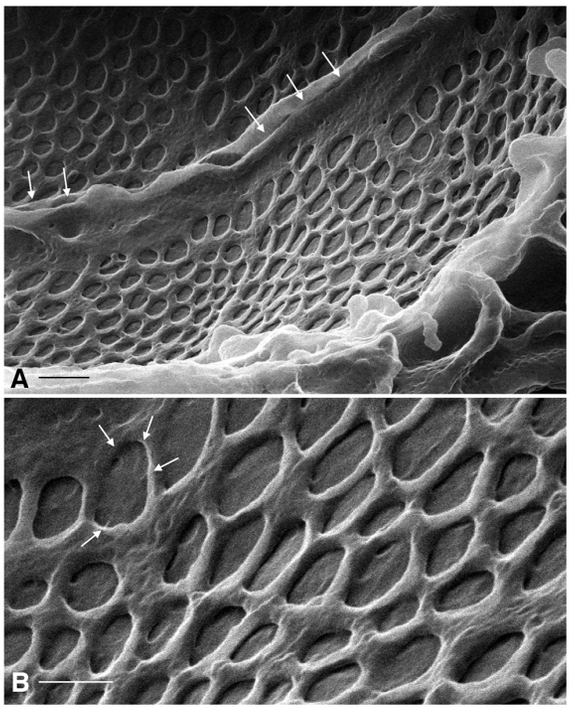

Figure 2. Images of glomerular endothelial cells as seen from the capillary luminal side using scanning helium ion microscopy. In image A, note the numerous, round fenestrations present over the entire cell surface. The raised ridges (arrows) represent the location of the tight junction between the two cells. Bar = 175 nm. In image B, under higher magnification, it is possible to observe the details of the fenestrations. In some of them, a substructure consisting of faint spokes like a bicycle wheel can be seen (arrows). Bar = 80 nm. Image courtesy of Prof. Dennis Brown [34], available at doi:10.1371/journal.pone.0057051.g004.

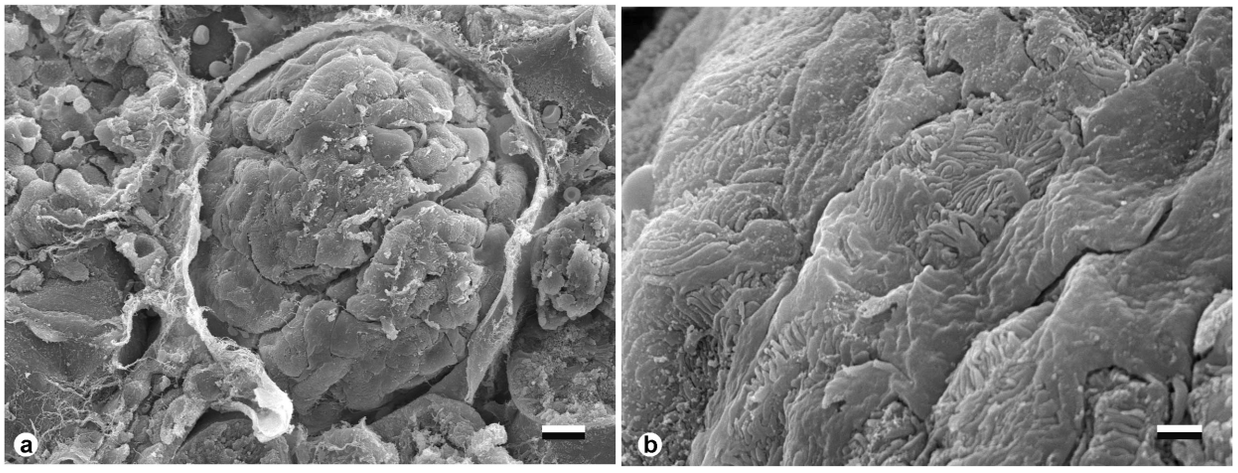

Figure 3. Scanning electron microscopic images of glomerulus of rats, showing the podocytes and its foot processes. In image A, under low magnification, we can observe the entire glomeruli with its Bowman's capsule (×950; 15 kV, scale bar represent 10 μm). In image B, we can observe several podocytes and foot processes (×6500; 15 kV, scale bar represent 1.5 μm).

interpretation due observer expertise [37]. Thus, it is clear that quantitative methods have advantages over those qualitative ones.

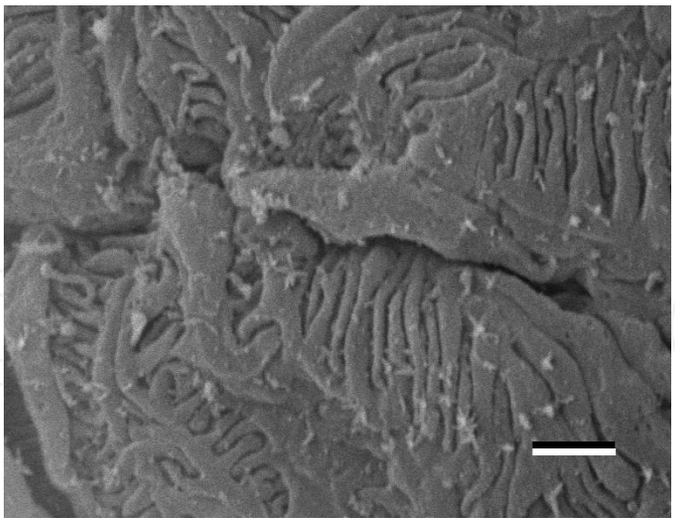

Figure 4. Scanning electron microscopic images showing the fine detail of the interdigitating pattern of the foot processes of podocytes at high magnification (×10,000; 15 kV, scale bar represent 1 μm).

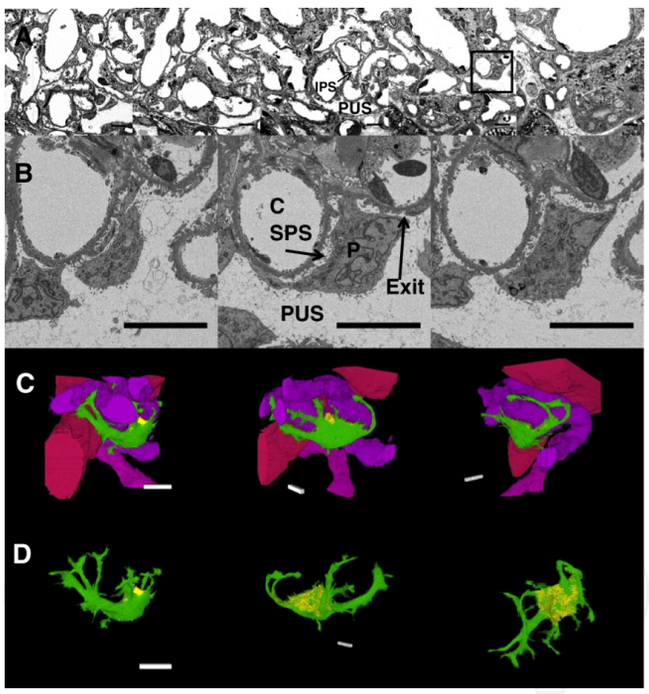

Figure 5. Scanning electron microscopy of a human glomerulus after serial block face slicing. In image A, we observe figures that resemble transmission electron micrographs by inverting the signal intensity of the backscattered electron. We see micrographs of complete field of view, with slices 15 μm apart one from another (*z*). In image B, we observe micrographs of a podocyte of a higher magnification of image A, illustrating the subpodocyte space. In image C, we observe the same podocyte as in image B and adjacent capillaries after highlighting and 3D reconstructing. Finally, in image D, the same podocytes of image C is separated from capillaries after 3D reconstruction, showing the subpodocyte space. P = podocytes, SPS = subpodocyte space, C = capillary lumen, PUS = periphery urinary space, IPS = interpodocyte space, and Exit is an exit region for an SPS. Purple/pink = capillaries, green = podocyte cell body, and yellow = SPS (under the main cell body). Scale bar is 10 μm in all cases. Image courtesy of Prof. Kenton Arkill [38], available at doi:10.1186/1471-2369-15-24.

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The glomerular basement membrane was recently observed in three dimensions by different methodologies [38]. In their paper, the authors compared the images and the 3D reconstruction of the glomerular filtration barrier as performed using serial block face scanning electron microscopy, focused ion beam milling scanning electron microscopy, and transmission electron tomography. They reported that the transmission electron tomography technique had the advantage of a higher resolution, with the disadvantages of limited field of view and anisotropic shrinkage. Focused ion bean and serial block face with scanning electron microscopy had greater field of view with lower resolution. The most interesting aspect of these techniques is the reconstruction and digital dissection of cells as demonstrated in Figure 5.

2.3.4. Quantitative analysis of the elements of the glomerular filtration barrier

The quantification of morphological structures is highly recommended for studying biological alterations in several tissues and different situations. The quantification of morphological aspects of the glomerular filtration barrier is possible and desirable. Translating the morphological aspects of tissues in numbers is very useful from a scientific point of view. It improves the understanding of the modifications of the barrier provoked by different conditions and allows statistical comparisons with other specimens, subjected to different conditions or at different stages of the disease [37]. Based on these premises, methods have been used for different purposes in the study of the glomerular filtration barrier morphology, and important scientific knowledge has been generated from these analyses.

Most quantitative analyses use digital microscopic images that can be digitally acquired or scanned from conventional images. For absolute values (linear measures, for example), it is very important that a scale is maintained on the image. Most electron microscopes automatically print a scale bar on each image, and this is very suitable for morphometric purposes [30].

Several software are eligible for morphometric analyses. In this chapter, we present the steps used in ImageJ 1.37v software. ImageJ is an open source, free software produced and distributed by the National Institutes of Health and can be downloaded at http://imagej.nih.gov/ij/index.html.

The different elements of endothelial layer of the glomerular filtration barrier can be objectively evaluated, and the possible loss of fenestrations can be easily assessed by linear measurements in transmission electron microscopy images. For this, we should first calibrate the software for the correct magnification. The straight-line tool should measure the scale bar of the image, and then we should use the option "analyze—set scale." In the box opened, the distance in pixels should represent the size of the line over the scale bar, and one should insert the real size of the bar at "known distance" space. The "unit of length" space should be fulfilled with the unit of the scale bar (μm or nm). When one intend to measure several images, all of them should be acquired under the same conditions (magnification, resolution, size, etc.), and the option "global" can be marked. By this, the same configuration may be used for all following micrographs. Figure 6 shows the above-mentioned steps.

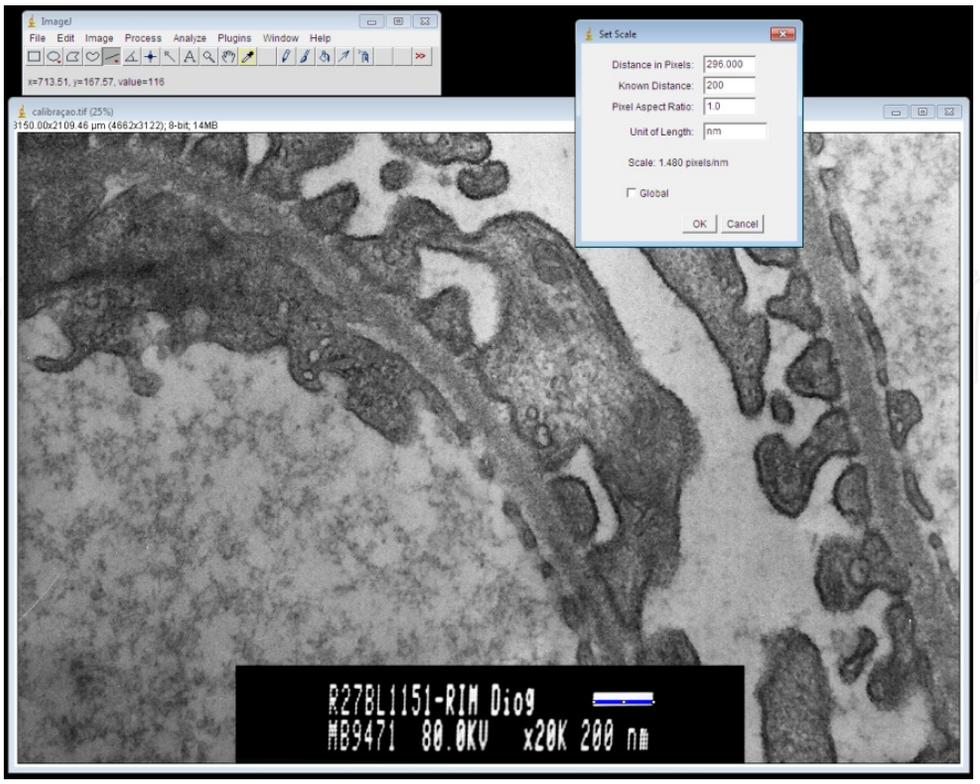

Figure 6. Transmission electron microscopy used to demonstrate how to calibrate the software for correct magnification of the micrograph with ImageJ software. The straight-line tool (in blue) is used to measure the scale bar, which here is 200 nm. Next, in the "set scale" box, one can see the distance in pixels of the blue line, and the space of "known distance" is fulfilled with 200 whereas "unit of length" with nm. After clicking "OK," any measurement in this image will be at nanometers calibrated for the real size.

After the software calibration for the magnification, the straight-line tool is used to measure ("Analyze—measure") the linear distance of endothelial cells and their fenestrations. The results, expressed in the unit of the scale bar, appear sequentially in the "results" window, and can be easily copied to spreadsheet or statistic software, as seen in Figure 7. One can further compare (a) the size of fenestrations, (b) the endothelial layer, and (c) their proportion (size of fenestrations/size of the endothelial layer). It is also possible to count the number of fenestrations observed on each image and divide by the linear size of the entire filtration barrier present on the image and thus obtain the number of fenestrations per distance. In addition, the thickness of endothelial cells can be measured by applying the straight-line perpendicular to basement membrane, in some randomly chosen points. One should consider that several measurements should be performed to obtain the whole thickness of endothelial layer, as it can greatly vary in the same glomerulus [39].

Furthermore, the glycocalyx that covers the endothelial cells can be studied with some special techniques. Because conventional methods do not stain the glycocalyx since it is mainly composed of proteoglycans, glycosaminoglycan, and hyaluronan, some techniques were developed for its identification. Perfusion with negatively charged lipid particles allows observation of these intralipids inside the capillary lumen. The measurement of linear distance from these intralipids to the endothelial inner membrane reflects glycocalyx thickness. When

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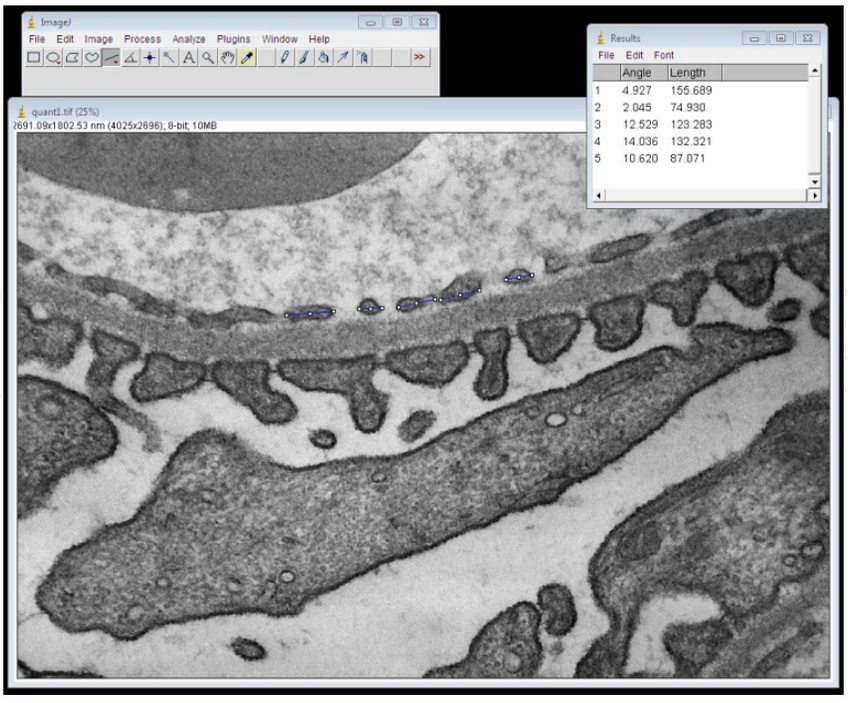

Figure 7. The linear distance of the endothelial cells can be measured with the straight-line tool of ImageJ software. In this electron micrograph, blue lines measure some endothelium segments, and the result is seen in the upper right corner. In the results box, the length of each measurement is expressed in nanometers because the image was previously calibrated for the real size.

reduction of glycocalyx thickness occurs, as measured by this method, correlation with proteinuria is thought, as this structure is one of the responsible for the filtration of plasma proteins [40,41].

Furthermore, the number of the endothelial cells per glomerulus can be measured using stereological methods. This needs determination of the glomerular volume (by the Cavalieri principle, dissector technique, or volume-weighted methods), the cellular density of glomerulus (counting cell nuclei by the Weibel and Gomez point-counting method), and the proportion of cell types of the glomerulus (also by the point-counting method) [40,41]. These measurements can be assessed with light microscopy with the benefits of a faster and cheaper method, or by electron microscopy with the advantage of being a more accurate method.

Regarding the basement membrane, despite its importance for the plasma filtration, the most commonly measurement performed is membrane width. This directly reflects the membrane thickness that occurs in several diseases and is easily and accurately assessed with transmission electron microscopy images. For this, after calibration for the magnification, the straight-line tool of ImageJ should be used perpendicularly to the membrane, as seen in Figure 8.

The effacement of the podocytes, which is commonly qualitatively assessed, can be objectively assessed by two methods [33]. In transmission electron microscopy images, the linear size of the foot processes touching and parallel to the basement membrane can be measured by the straight-line tool, as shown in Figure 9a. As the podocyte effacement is characterized by the loss of the normal interdigitating pattern, with fewer and larger foot processes, in this

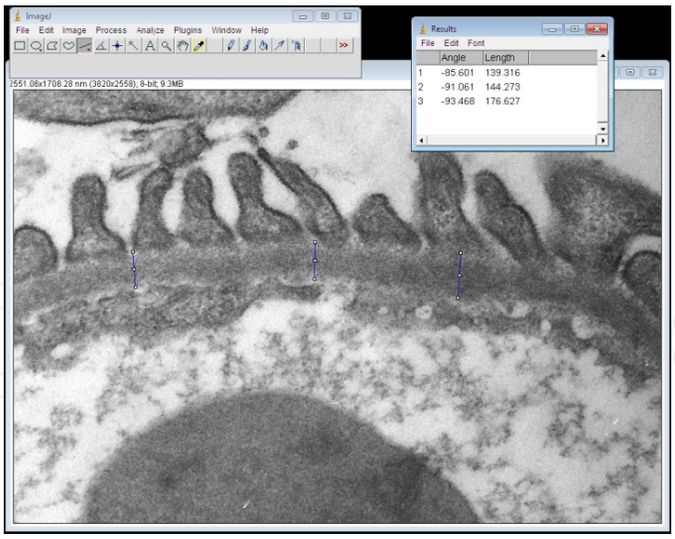

Figure 8. The basement membrane width easily measured in transmission electron micrographs using the "straight line" tool of the ImageJ software. In this image, blue lines measure the basement membrane width, and the result is seen in upper right corner of the image. After calibration of the software, each basement membrane measurement is expressed in nanometers in the results box.

condition, these processes present a higher area touching the basement membrane. Thus, the increased linear size measured by this method numerically represents the podocyte effacement. In addition, one can determine the number of slit diaphragm divided by the linear size of the entire filtration barrier present on the image, and the number of slit diaphragm per distance is obtained (Figure 9b), which is also reduced in podocyte effacement. Also, the number of foot processes per distance can be measured by the same method, as used by Jonsson et al. [42], who quantitatively demonstrated the podocytes effacement, as observed in Figure 10. The size of slit diaphragm can be also easily measured using the straight-line tool applied parallel to the basement membrane, after software calibration.

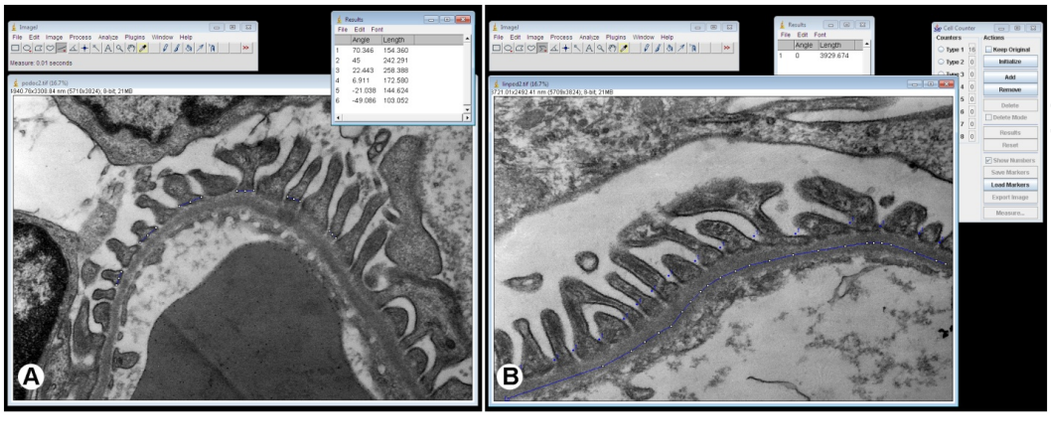

Figure 9. Measurement of foot process parameters that relate with podocyte effacement using transmission electron micrographs. In image A, the linear size of the foot processes that touch the basement membrane is measured by the straight-line tool. Several foot processes can be measured for this parameter. In image B, slit diaphragms can be counted using the "cell counter" tool of ImageJ and the linear size of basement membrane, measured with the "Segmented line selections" tool.

http://dx.doi.org/10.5772/61811

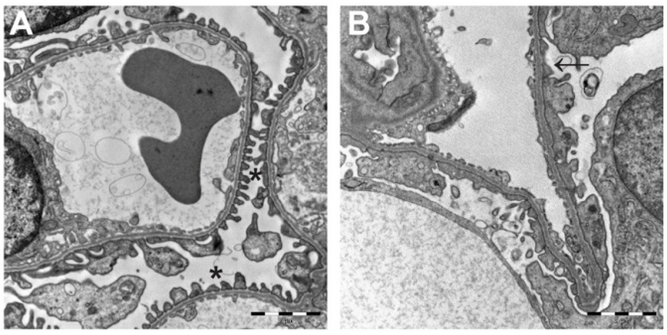

Figure 10. Transmission electron microscopic images of control mice and animals with passive Heymann nephritis. In image A, we see the normal glomerular filtration barrier (*) in control animals. In image B, from mice that received an injection of Anti-Fx1A IgG antibody to induce passive Heymann nephritis, it is possible to observe a high degree of foot process effacement (arrow). Scale bar = 2 mm. Image courtesy of Prof. Annika Lindskog Jonsson [42], available at doi:10.1371/journal.pone.0087816.g004.

In scanning electron microscopy images, the number of foot processes per podocyte can also be measured. This parameter only could be determined in podocytes that can be entirely visualized in the scanning image, and special attention should be taken for not count the foot process of the adjacent cell. This makes this method time consuming but can be an option when only scanning electron microscopy is available. In addition, the size of the foot process can be measured in these images, however, with fewer accuracy than when measured in transmission electron microscopy images (Figure 11). Finally, the number of podocytes per glomerulus can be determined using the same method described for determination of endothelial cells per glomerulus.

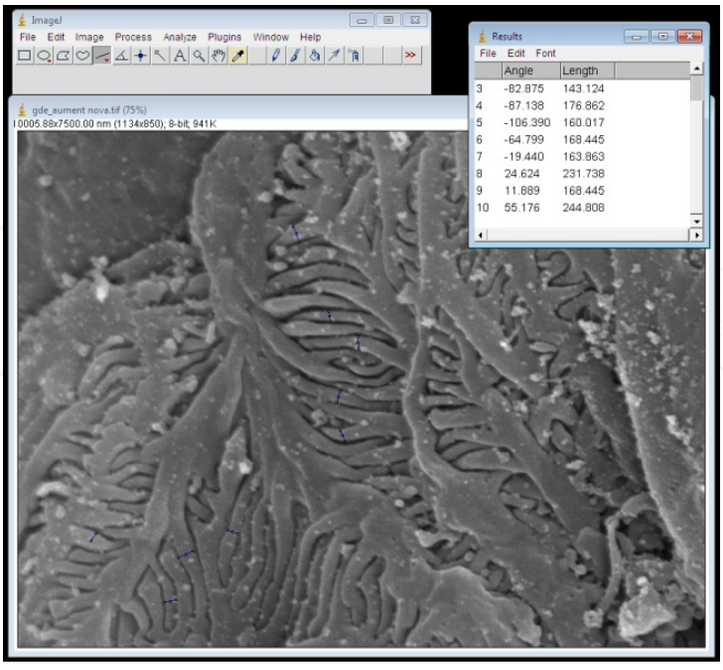

Figure 11. The foot processes can also be measured in scanning electron micrographs with the straight-line tool of ImageJ software after calibration for the correct magnification.

3. Conclusions

The glomerular filtration barrier is a main component for the filtration of the plasma and formation of primary urine. It is composed of specialized cells and noncellular structures that, together, can avoid the loss of important plasma components but permit the passage of water and undesirable molecules. For this functionality, this barrier has a specific morphology with a fenestrated endothelium covered with glycocalyx, a basement membrane, and a set of slit diaphragms formed by the foot processes of podocytes.

The glomerular filtration barrier morphology has been studied in several diseases and is directly associated with kidney malfunction. Furthermore, it is very important to study this barrier under different clinical and experimental situations. Morphological alterations of components explain some physiopathological findings in clinical setting and correlate with kidney function. For this, scanning and transmission electron microscopy suits perfectly for obtaining high-quality images of this barrier.

Several studies qualitatively described the alterations of glomerular filtration membrane. This gives valuable information, especially when reporting clinical cases or individual lesions that could not be compared. However, when the purpose of the study is to compare the results of a group of individuals with another, quantitative analysis is more appropriate. In this chapter, we presented some objective methods for easy evaluation glomerular filtration membrane. Results obtained with these methods generate numerical data that can be statistically compared with other groups, in different phases of the disease, after some treatment, etc. Thus, whenever possible, quantitative analysis of the glomerular filtration barrier should be favored.

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References

[1] Bloom W, Fawcett DW. A Textbook of Histology. 10th ed. Philadelphia: Saunders; 1975.

[2] Jarad G, Miner JH. Update on the glomerular filtration barrier. Curr Opin Nephrol Hypertens. 2009;18:226–232. DOI: 10.1097/MNH.0b013e3283296044

[3] Mundel P, Kriz W. Structure and function of podocytes: an update. Anat Embryol (Berl). 1995;192:385–397. DOI: 10.1007/BF00240371

[4] Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004;27:1047–1053. DOI: 10.2337/diacare.27.5.1047

[5] Mould AP, Craig SE, Byron SK, Humphries MJ, Jowitt TA. Disruption of integrin-fibronectin complexes by allosteric but not ligand-mimetic inhibitors. Biochem J. 2014;464:301–313. DOI: 10.1042/BJ20141047

[6] Relle M, Cash H, Brochhausen C, Strand D, Menke J, Galle PR, et al. New perspectives on the renal slit diaphragm protein podocin. Mod Pathol. 2011;24:1101–1110. DOI: 10.1038/modpathol.2011.58

[7] Inoue K, Ishibe S. Podocyte endocytosis in the regulation of the glomerular filtration barrier. Am J Physiol Renal Physiol. 2015;309:F398-F405. DOI: 10.1152/ajprenal.00136.2015

[8] Lennon R, Randles MJ, Humphries MJ. The importance of podocyte adhesion for a healthy glomerulus. Front Endocrinol (Lausanne). 2014;5:160. DOI: 10.3389/fendo.2014.00160

[9] Craici IM, Wagner SJ, Weissgerber TL, Grande JP, Garovic VD. Advances in the pathophysiology of pre-eclampsia and related podocyte injury. Kidney Int. 2014;86:275–285. DOI: 10.1038/ki.2014.17

[10] Gaede P, Lund-Andersen H, Parving HH, Pedersen O. Effect of a multifactorial intervention on mortality in type 2 diabetes. N Engl J Med. 2008;358:580–591. DOI: 10.1056/NEJMoa0706245

[11] Kannel WB, Stampfer MJ, Castelli WP, Verter J. The prognostic significance of proteinuria: the Framingham study. Am Heart J. 1984;108:1347–1352. DOI: 10.1016/0002-8703(84)90763-4

[12] Yuyun MF, Dinneen SF, Edwards OM, Wood E, Wareham NJ. Absolute level and rate of change of albuminuria over 1 year independently predict mortality and cardiovascular events in patients with diabetic nephropathy. Diabet Med. 2003;20:277–282. DOI: 10.1046/j.1464-5491.2003.00940.x

[13] Schena FP, Gesualdo L. Pathogenetic mechanisms of diabetic nephropathy. J Am Soc Nephrol. 2005;16 Suppl 1:S30–33. DOI: 10.1681/ASN.2004110970

[14] Tryggvason K, Patrakka J, Wartiovaara J. Hereditary proteinuria syndromes and mechanisms of proteinuria. N Engl J Med. 2006;354:1387–1401. DOI: 10.1056/NEJMra052131

[15] Pistoia F, Sacco S, Degan D, Tiseo C, Ornello R, Carolei A. Hypertension and stroke: epidemiological aspects and clinical evaluation. High Blood Press Cardiovasc Prev. DOI: 10.1007/s40292-015-0115-2

[16] Bidani AK, Griffin KA. Long-term renal consequences of hypertension for normal and diseased kidneys. Curr Opin Nephrol Hypertens. 2002;11:73–80. DOI: 10.1097/00041552-200201000-00011

[17] Lan HY. Diverse roles of TGF-beta/Smads in renal fibrosis and inflammation. Int J Biol Sci. 2011;7:1056–1067. DOI: 10.7150/ijbs.7.1056

[18] Kato T, Mizuguchi N, Ito A. Blood pressure, renal biochemical parameters and histopathology in an original rat model of essential hypertension (SHRSP/Kpo strain). Biomed Res. 2015;36:169–177. DOI: 10.2220/biomedres.36.169

[19] Le Jemtel TH, Rajapreyar I, Selby MG, Payne B, Barnidge DR, Milic N, et al. Direct evidence of podocyte damage in cardiorenal syndrome type 2: preliminary evidence. Cardiorenal Med. 2015;5:125–134. DOI: 10.1159/000375130

[20] 20. Barker DJ, Forsen T, Uutela A, Osmond C, Eriksson JG. Size at birth and resilience to effects of poor living conditions in adult life: longitudinal study. BMJ. 2001;323:1273–1276. DOI: 10.1136/bmj.323.7324.1273

[21] Gregorio BM, Souza-Mello V, Carvalho JJ, Mandarim-de-Lacerda CA, Aguila MB. Maternal high-fat intake predisposes nonalcoholic fatty liver disease in C57BL/6 offspring. Am J Obstet Gynecol. 2010;203:495 e1–8. DOI: 10.1016/j.ajog.2010.06.042

[22] Hoy WE, Douglas-Denton RN, Hughson MD, Cass A, Johnson K, Bertram JF. A stereological study of glomerular number and volume: preliminary findings in a multiracial study of kidneys at autopsy. Kidney Int Suppl. 2003;63:S31-S37. DOI: 10.1046/j.1523-1755.63.s83.8.x

[23] Ikezumi Y, Suzuki T, Karasawa T, Yamada T, Hasegawa H, Nishimura H, et al. Low birthweight and premature birth are risk factors for podocytopenia and focal segmental glomerulosclerosis. Am J Nephrol. 2013;38:149–157. DOI: 10.1159/000353898

[24] Sene Lde B, Mesquita FF, de Moraes LN, Santos DC, Carvalho R, Gontijo JA, et al. Involvement of renal corpuscle microRNA expression on epithelial-to-mesenchymal transition in maternal low protein diet in adult programmed rats. PLoS One. 2013;8:e71310. DOI: 10.1371/journal.pone.0071310

[25] Hodgin JB, Rasoulpour M, Markowitz GS, D'Agati VD. Very low birth weight is a risk factor for secondary focal segmental glomerulosclerosis. Clin J Am Soc Nephrol. 2009;4:71–76. DOI: 10.2215/CJN.01700408

[26] Villar-Martini VC, Carvalho JJ, Neves MF, Aguila MB, Mandarim-de-Lacerda CA. Hypertension and kidney alterations in rat offspring from low protein pregnancies. J Hypertens Suppl. 2009;27:S47-S51. DOI: 10.1097/01.hjh.0000358838.71675.5e

[27] Lapillonne A. Vitamin D deficiency during pregnancy may impair maternal and fetal outcomes. Med Hypotheses. 2010;74:71–75. DOI: 10.1016/j.mehy.2009.07.054

[28] Nascimento FA, Ceciliano TC, Aguila MB, Mandarim-de-Lacerda CA. Maternal vitamin D deficiency delays glomerular maturity in F1 and F2 offspring. PLoS One. 2012;7:e41740. DOI: 10.1371/journal.pone.0041740

[29] Maka N, Makrakis J, Parkington HC, Tare M, Morley R, Black MJ. Vitamin D deficiency during pregnancy and lactation stimulates nephrogenesis in rat offspring. Pediatr Nephrol. 2008;23:55–61. DOI: 10.1007/s00467-007-0641-9.

[30] Maunsbach AB, Afzelius BA. Biomedical Electron Microscopy. Illustrated Method and Interpretations. London: Academic Press; 1999.

[31] Souza W. Técnicas de microscopia eletrônica aplicadas às ciências biológicas. 3rd ed. Rio de Janeiro: Soc. Bras. Microsc; 2007.

[32] Bone Q, Denton EJ. The osmotic effects of electron microscope fixatives. J Cell Biol. 1971;49:571–581. DOI: 10.1083/jcb.49.3.571

[33] Souza DB, Costa WS, Cardoso LE, Benchimol M, Pereira-Sampaio MA, Sampaio FJ. Does prolonged pneumoperitoneum affect the kidney? Oxidative stress, stereological and electron microscopy study in a rat model. Int Braz J Urol. 2013;39:30–36. DOI: 10.1083/jcb.49.3.571

[34] 34. Rice WL, Van Hoek AN, Paunescu TG, Huynh C, Goetze B, Singh B, et al. High resolution helium ion scanning microscopy of the rat kidney. PLoS One. 2013;8: e57051. DOI:10.1371/journal.pone.0057051

[35] Shirato I. Podocyte process effacement in vivo. Microsc Res Tech. 2002;57:241–246. DOI: 10.1002/jemt.10082

[36] Wagner MC, Rhodes G, Wang E, Pruthi V, Arif E, Saleem MA, et al. Ischemic injury to kidney induces glomerular podocyte effacement and dissociation of slit diaphragm proteins Neph1 and ZO-1. J Biol Chem. 2008;283:35579–35589. DOI: 10.1074/jbc.M805507200

[37] Felix-Patricio B, De Souza DB, Gregório BM, Costa WS, Sampaio FJ. How to quantify penile corpus cavernosum structures with histomorphometry: comparison of two methods. Biomed Res Int. DOI: 10.1155/2015/832156

[38] Arkill KP, Qvortrup K, Starborg T, Mantell JM, Knupp C, Michel CC, et al. Resolution of the three dimensional structure of components of the glomerular filtration barrier. BMC Nephrol. 2014;15:24. DOI: 10.1186/1471-2369-15-24

[39] Hjalmarsson C, Johansson BR, Haraldsson B. Electron microscopic evaluation of the endothelial surface layer of glomerular capillaries. Microvasc Res. 2004;67:9–17. DOI: 10.1016/j.mvr.2003.10.001

[40] Andersson M, Nilsson U, Hjalmarsson C, Haraldsson B, Nystrom JS. Mild renal ischemia-reperfusion reduces charge and size selectivity of the glomerular barrier. Am J Physiol Renal Physiol. 2007;292:F1802-F1809. DOI: 10.1152/ajprenal.00152.2006

[41] Steffes MW, Schmidt D, McCrery R, Basgen JM. Glomerular cell number in normal subjects and in type 1 diabetic patients. Kidney Int. 2001;59:2104–2113. DOI: 10.1046/j.1523-1755.2001.00725.x

[42] Jonsson AL, Granqvist A, Elvin J, Johansson ME, Haraldsson B, Nystrom J. Effects of melanocortin 1 receptor agonists in experimental nephropathies. PLoS One. 2014;9:e87816. DOI: 10.1371/journal.pone.0087816