The Convoluted Tubules of the Nephron Must Be Considered Elliptical, and Not Circular, in Stereological Studies of the Kidney

Marta Ortega-Martinez, Vanessa Gutierrez-Davila, Esthefania Gutierrez-Arenas, Alberto Niderhauser-Garcia, Ricardo M. Cerda-Flores, Gilberto Jaramillo-Rangel

Department of Pathology, School of Medicine, Autonomous University of Nuevo Leon, Monterrey, Mexico; School of Biology, Autonomous University of Sinaloa, Culiacan, Mexico; School of Nursing, Autonomous University of Nuevo Leon, Monterrey, Mexico

Keywords
Distal convoluted tubule · Kidney · Mouse · Proximal convoluted tubule

Abstract
Introduction: The diameter and area of the proximal convoluted tubule (PCT) and the distal convoluted tubule (DCT) are of the main parameters analyzed in stereological studies of the kidney. However, there is no consensus about if the PCT and DCT should be considered circular or elliptical in shape. Objective: To analyze if there are significant differences in the diameter and area of the PCT and DCT, depending on whether they are considered circular or elliptical. Methods: Paraffin-embedded sections of kidneys from CD1 mice were stained with hematoxylin and eosin and examined using a light microscope. Images were captured using a camera linked to image analysis software. A short diameter (d) and a long diameter (D) were measured in both PCT and DCT. A small circular area (SCA), a large circular area (LCA), and an elliptical area (EA) were calculated with mathematical formulas that incorporate d and D values, while a program area (PA) was provided by the software. Results: There was a significant difference between d and D in both PCT (F = 1.354, Sig = 0.000) and DCT (F = 4.989, Sig = 0.000). Also, there were significant differences in the tubular areas in both PCT (F = 34.843, Sig = 0.000) and DCT (F = 22.390, Sig = 0.000); circular areas were different from elliptical areas (SCA and LCA vs. EA and PA). Conclusion: The convoluted tubules of the nephron must not be considered circular, but rather elliptical; care should be taken every time the tubules are analyzed in stereological studies of the kidney, especially when evaluating their diameters and areas.

Introduction
Morphometry is the term applied to the measurement of shape or structure. Stereology, sometimes used synonymously, refers to mathematical methods based on geometric probability that allow to measure three-dimensional characteristics from two-dimensional images [1].

Stereological methods have been used to examine how the kidney reacts to trauma, disease, or experimental conditions. Exact knowledge of the number, size, and distribution of the kidney components in those situations provides important information about its organization and function [2].
In addition to the glomerulus, the proximal convoluted tubule (PCT) and the distal convoluted tubule (DCT) are the most widely analyzed structures in stereological kidney research. In turn, the diameter and less frequently the area are the main measurements evaluated in the PCT and DCT. However, there is no consensus in the literature on how tubular diameter (or diameters) should be evaluated. Several authors measure only one diameter, as if the tubules were circular [3–10]. On the contrary, other authors consider that the tubules have an elliptical shape and report the short diameter (minimum, proximal), sometimes along with the large diameter (maximum, distal) [11–20]. Even more, other authors measure in the same sample some tubules as circles and others as ellipses [21, 22]. Besides, the calculation of the tubular area may depend on the way the diameter is evaluated [10, 15].

Other approaches to the study of the nephron tubules also do not consider whether they are circular or elliptical. Since the in vivo analysis of the function of the tubules is significantly difficult, mathematical models have been created, but they only consider tubules as circular cylinders [23, 24]. Interestingly, fractal analysis of nephron tubules in paraffin-embedded sections stained with routine histological methods has revealed that they are, in fact, fractals [25, 26]. Since a circle is a nonfractal structure [27], tubules could have another shape. The purpose of this work was to analyze if there are significant differences in the diameter and area of PCT and DCT, depending on whether they are considered circular or elliptical in the stereological analysis.

**Materials and Methods**

**Animals and Experimental Design**

Animals used were described in previous papers, in which stereological methods were employed to examine the lung [28, 29]. Male CD1 mice were maintained in standard conditions in stainless steel cages (temperature: 18–21°C; relative humidity: 55–60%; circadian rhythm: 12 h of artificial light per day, 12 h of darkness per day). The standard chow and water were available ad libitum. Three animals were sacrificed at the age of 2 months by cervical dislocation. Only the right-side kidneys were processed and analyzed in order to have a more consistent control in the selection of samples. Kidneys were divided into 2 equal halves longitudinally, fixed by immersion in 10% neutral buffered formalin, and embedded in paraffin with the samples oriented with the flat surface parallel to the surface of the paraffin block to be sectioned. Of note, although some authors who analyzed the renal tubules mor-
phometrically fixed the kidneys by perfusion before inclusion in paraffin [4, 11, 22], most used the immersion method for this purpose [6, 8–10, 12–21]. Then, 5-μm thick sections were cut, deparaffinized in xylene, hydrated in a graded series of alcohol, and stained with hematoxylin and eosin (H&E) according to standard techniques.

Analysis was conducted using 3 tissue sections per animal. In each slide, 20 PCTs and 20 DCTs were examined in randomly selected microscopic fields at ×400 magnification. Thus, a total of 120 tubular structures were analyzed per animal. It is noteworthy that in the literature, some authors do not specify the total number of tubules examined per subject [5, 6, 8, 9, 11, 20]. When specified, most authors report measurements in 100 tubules or fewer [3, 4, 10, 12–18, 21, 22]. Only transverse tubules with complete basement membrane (BM) were evaluated; longitudinal tubules were not considered.

Morphometry

Sections were examined with a light microscope (Primo Star; Carl Zeiss Microscopy GmbH, Oberkochen, Germany), and high-resolution color images were digitalized onto a computer screen using a AxioCam ICc1 camera (Carl Zeiss Microscopy GmbH) linked to image analysis software (Zen lite 2011; Carl Zeiss Microscopy GmbH). All the sections were examined on coded slides by a single observer.

The parameters analyzed in both PCT and DCT are illustrated in Figures 1–3. The short diameter (d) was defined as the length of the shortest straight line that passes through the center of the tubule and connects 2 opposite extreme points of the BM. The large diameter (D) was defined as the length of the largest straight line that passes through the center of the tubule and connects 2 opposite extreme points of the BM. To measure both diameters, lines d and D were manually drawn on the captured images described above, and the length of the lines was provided by the software.

The tubular area was defined as the space bounded by the BM. The small circular area (SCA) was calculated by using the formula for the area of a circle, \( A = \pi r^2 \), where \( r \) is one-half of \( d \). The large circular area (LCA) was calculated by using the same formula, but replacing the \( d \) value by \( D \).

The elliptical area (EA) was obtained by using the formula for the area of an ellipse, \( A = \pi \cdot d' \cdot D' \), where \( d' \) and \( D' \) are one-half of \( d \) and \( D \). Finally, the program area (PA) was obtained using the software mentioned above. To measure it, a line was drawn manually through the entire tubular perimeter, and the area was provided by the software.

Fig. 2. Morphometric analysis of a PCT. Representative kidney tissue section from a CD1 mouse stained with hematoxylin and eosin. The short (d) and large (D) diameters are exemplified. The area was the space delimited by the blue line. The PA is exemplified. The other 3 areas, SCA, LCA, and EA, were obtained with mathematical formulas using the values of \( d \) and \( D \). Bar = 20 μm. G, glomerulus; BV, blood vessel; PCT, proximal convoluted tubule; SCA, small circular area; LCA, large circular area; EA, elliptical area; PA, program area.

Fig. 3. Morphometric analysis of a DCT. Representative kidney tissue section from a CD1 mouse stained with hematoxylin and eosin. The short (d) and large (D) diameters are exemplified. The area was the space delimited by the blue line. The PA is exemplified. The other 3 areas, SCA, LCA, and EA, were obtained with mathematical formulas using the values of \( d \) and \( D \). Bar = 20 μm. G, glomerulus; DCT, distal convoluted tubule; SCA, small circular area; LCA, large circular area; EA, elliptical area; PA, program area.
Statistical Analysis

The results are presented as means ±1 standard error. Student’s t test was used to determine statistical significance between the tubular diameters (\(d\) and \(D\)). Comparisons between the tubular areas (SCA, LCA, EA, and PA) were made by a one-way ANOVA, followed by post hoc analysis using the least significant difference (LSD) test. The significance level for all tests was set at \(p < 0.05\). The data were analyzed using the SPSS for Windows software (SPSS, Inc., Chicago, IL, USA), release 21.0.

Results

The results are summarized in Tables 1 and 2. Student’s t test showed a significant difference between the diameters analyzed in both PCT (\(F = 1.354, \text{Sig} = 0.000\)) and DCT (\(F = 4.989, \text{Sig} = 0.000\)). In the PCT (Fig. 4), \(D\) (44.0 ± 0.7 μm) was significantly greater in comparison with \(d\) (37.4 ± 0.5 μm). Similarly, in the DCT (Fig. 4), \(D\) (40.2 ± 0.9 μm) was significantly greater when compared to \(d\) (32.2 ± 0.6 μm).

The ANOVA test showed significant differences in the tubular areas analyzed in both PCT (\(F = 34.843, \text{Sig} = 0.000\)) and DCT (\(F = 22.390, \text{Sig} = 0.000\)). The LSD test revealed that in the PCT (Fig. 5), LCA (1,543 ± 47 μm²) was significantly greater than SCA, EA, and PA (1,112 ± 31, 1,300 ± 33, and 1,097 ± 25 μm², respectively; all \(p\) values of 0.000). Also, EA was significantly greater when compared to SCA and PA (\(p\) values of 0.000).

The LSD test revealed that in the DCT (Fig. 5), LCA (1,313 ± 69 μm²) was significantly greater than SCA, EA, and PA (829 ± 29, 1,027 ± 35, and 976 ± 20 μm², respectively; all \(p\) values of 0.000). EA was significantly greater when compared to SCA (\(p\) value of 0.001). Finally, PA was significantly greater than SCA (\(p\) value of 0.016).

Discussion

The diameter and the area of PCT and DCT are some of the main parameters analyzed in stereological studies of the kidney. The results obtained in this work indicate that the accuracy and reproducibility of those analyses may depend on how the shape of the tubules is considered.

Several authors considered that the tubules have a circular shape and therefore measured only one diameter [3–10]. However, our results showed that the convoluted tubules of the kidney are elliptical in shape; we measured a short diameter (the short axis of the ellipse) and a long diameter (the long axis of the ellipse) which differed significantly from each other (Table 1; Fig. 4). Therefore, when analyzing the tubules in stereological studies of the

### Table 1. Diameters (μm) of the convoluted tubules

|                  | Short diameter | Large diameter | \(p\) values |
|------------------|----------------|----------------|-------------|
| Proximal convoluted tubules | 37.4±0.5       | 44.0±0.7       | 0.000       |
| Distal convoluted tubules     | 32.2±0.6       | 40.2±0.9       | 0.000       |

Values are reported as mean±1 standard error. Data were analyzed by Student’s t test. The results were considered statistically significant at \(p < 0.05\).

### Table 2. Areas (μm²) of the convoluted tubules

|                  | SCA          | LCA          | EA           | PA           | \(p\) values |
|------------------|--------------|--------------|--------------|--------------|-------------|
| Proximal convoluted tubules | 1,112±31     | 1,543±47     | 1,300±33     | 1,097±25     | 0.000a, b   |
| Distal convoluted tubules     | 829±29       | 1,313±69     | 1,027±35     | 976±20       | 0.000c       |

Values are reported as mean±1 standard error. Data were analyzed by a one-way ANOVA followed by a least significant difference test. SCA, small circular area; LCA, large circular area; EA, elliptical area; PA, program area. The results were considered statistically significant at \(p < 0.05\). a LCA versus SCA, EA, and PA. b EA versus SCA and PA. c LCA versus SCA, EA, and PA. d EA versus SCA. e PA versus SCA.
kidney, it should be considered that if only one diameter of the tubules is measured and a significant difference is observed, it may be due to the fact that both axes of the ellipse were actually measured, either randomly or alternatively, and not to the treatment or phenomenon analyzed per se. Furthermore, it can be difficult to reproduce a study when the shape of the tubules is considered circular because any axis of the ellipse, or both in a random way, could be measured in the replication study.

These problems may not arise if the tubules are considered elliptical and the same axis (short or long) is evaluated in all tubules. Most authors report the value of the
short axis (short diameter) of the ellipse [12–14, 16–19], while others report the value of both axes (short and long diameters) of the ellipse [11, 15, 20]. Furthermore, care should be taken to always measure tubules as ellipses and not some tubules as ellipses and other tubules as circles in the same sample [21, 22].

The tubules certainly have an elliptical shape. We were able to calculate an EA with a formula that incorporates the values of a short axis and a long axis, and the line used to measure the area with the software (PA) clearly delimited an ellipse (Fig. 2, 3).

The SCA and the LCA differed significantly from each other in both PCT and DCT (Table 2; Fig. 5). SCA and LCA were calculated using the value of the short diameter and the value of the large diameter of the circle, respectively, and this fact would explain the difference encountered. Therefore, observations regarding accuracy and reproducibility described above for the circular diameters could also be applied to the circular area analysis.

Interestingly, significant differences were also observed between other areas evaluated. In the PCT, LCA was also significantly different from EA and PA, while EA was significantly different from SCA and PA (Table 2; Fig. 5). In the DCT, LCA was also significantly different from EA and PA, while EA and PA were significantly different from SCA (Table 2; Fig. 5). It is likely that the area of a tubule may vary not only depending on whether it is considered a circle or an ellipse (SCA and LCA vs. EA and PA) but also if the area is evaluated directly with a piece of software or with another strategy (PA vs. EA). While the use of image analysis software to assess morphometric data might be more exact in comparison with other strategies, it should be remembered that it is the researcher who ultimately provides the necessary information to the software for the correct execution of the measurements [30]. In this case, the software would be of little use if the shape of the tubules were considered circular instead of elliptical.

Finally, although recommendations have been made for the morphometric analysis of the kidney [2], there is no consensus in the literature on how to perform it regarding several aspects, such as which method for the fixation of the kidney is the correct one, the correct orientation of the samples for histological analysis, the number of structures that should be evaluated, the optimal sampling strategy, and if the nephron tubules should be considered circular or elliptical. With this work, we try to clarify the last-mentioned aspect. In conclusion, the results obtained in this work indicate that the convoluted tubules of the nephron must not be considered circular, but rather elliptical, and care should be taken every time the tubules are analyzed in stereological studies of the kidney, especially when evaluating their diameters and areas.

**Statement of Ethics**

The present study was performed in accordance with the principles and procedures defined in the National Research Council Guide for the Care and Use of Laboratory Animals (8th edition) and in the Mexican Guidelines ZOO-062. Experimental protocols were approved by the Institutional Review Board and Ethics Committee of the School of Medicine of the Autonomous University of Nuevo Leon (No. PA19-00001).

**Conflict of Interest Statement**

The authors declare that they have no conflicts of interest.

**Funding Sources**

This research received no external funding.

**Author Contributions**

M.O.-M. and V.G.-D. performed experiments. G.J.-R. planned experiments. M.O.-M. and E.G.-A. provided the image analysis. G.J.-R., M.O.-M., and E.G.-A. wrote the manuscript. R.M.C.-F. provided the statistical analysis of the data. G.J.-R. conceived and designed the study. A.N.-G. performed the analysis and interpretation of data. All authors read and approved the manuscript.

**References**

1 Hamilton PW, Allen DC. Morphometry in histopathology. J Pathol. 1995 Apr; 175(4): 369–79.
2 Nyengaard JR. Stereologic methods and their application in kidney research. J Am Soc Nephrol. 1999 May; 10(5): 1100–23.
3 Hayslett JP, Kashgarian M, Epstein FH. Changes in proximal and distal tubular reabsoption produced by rapid expansion of extracellular fluid. J Clin Invest. 1967 Jul;46(7): 1254–63.
4 Davis RG, Madsen KM, Fregly MJ, Tisher CC. Kidney structure in hypothyroidism. Am J Pathol. 1983 Oct;113(1): 41–9.
5 Lebrecht D, Venhoff AC, Kirschner I, Wiech T, Venhoff N, Walker UA. Mitochondrial tubulopathy in tenofovir disoproxil fumarate-treated rats. J Acquir Immune Defic Syndr. 2009 Jul;51(3):258–63.
6 Saraga M, Vukojević K, Krželj V, Puretić Z, Bočina I, Durdov MG, et al. Mechanism of cystogenesis in nephrotic kidneys: a histopathological study. BMC Nephrol. 2014 Jan; 15:3.
7 Hemmi S, Matsumoto N, Jike T, Obana Y, Nakanishi Y, Soma M, et al. Proximal tubule morphology in rats with renal congestion: a study involving the in vivo cryotechnique. Med Mol Morphol. 2015 Jun;48(2):92–103.
8 Amjad Z, Yasmin T, Perveen K, Miranda T, Shoro AA. Lead-induced morphometric changes in the kidneys of albino rats ameliorated by ginkgo biloba extract (EGb 761). J Pak Med Assoc. 2017 Jan;67(1):58–65.
9 Momeni HR, Eskandari N. Effect of curcumin on kidney histopathological changes, lipid peroxidation and total antioxidant capacity of serum in sodium arsenite-treated mice. Exp Toxicol Pathol. 2017 Feb;69(2):93–7.
10 Truter D, Chellán N, Strijdom H, Webster I, Rawstoner J, Kotzé SH. Histomorphological changes in the pancreas and kidney and histopathological changes in the liver in male Wistar rats on antiretroviral therapy and melatonin treatment. Acta Histochem. 2018 May; 120(4):347–55.
11 Welling LW, Welling DJ. Shape of epithelial cells and intercellular channels in the rabbit proximal nephron. Kidney Int. 1976 May; 9(5):385–94.
12 Okada K, Takahashi S. Measurement of proximal tubular diameter for evaluation of tubular hypertrophy. Nephron. 1995;70(1):122.
13 Fujimaki M, Nagase M, Uchida S. Long-term effect of manidipine on renal function and structure in uninephrectomized spontaneously hypertensive rats. Clin Exp Pharmacol Physiol. 1997 Jul;24(7):506–12.
14 Lane PH. Long-term furosemide treatment in the normal rat: dissociation of glomerular hypertrophy and glomerulosclerosis. Am J Kidney Dis. 1999 Jun;33(6):1058–63.
15 Czekaj P, Palasz A, Lebda-Wyborny T, Nowaczek-Dura G, Karczewski W, Florok E, et al. Morphological changes in lungs, placenta, liver and kidneys of pregnant rats exposed to cigarette smoke. Int Arch Occup Environ Health. 2002 Oct;75 Suppl:S27–35.
16 Sagen JV, Bostad L, Njølstad PR, Søvik O. Enlarged nephrons and severe non-diabetic nephropathy in hepatocyte nuclear factor-1β (HNF-1β) mutation carriers. Kidney Int. 2003 Sep;64(3):793–800.
17 Okada K, Matsumoto K. Effect of dietary salt restriction on tubular hypertrophy in rats with early-stage chronic renal failure. Scand J Urol Nephrol. 2004;38(4):326–31.
18 Okada K, Matsumoto K. Oral ascorbend prevents reduction of anionic sites of the glomerular basement membrane in diabetic nephropathy. Nephron Exp Nephrol. 2005;99(2):e56–62.
19 Leh S, Hulfström M, Rosenberger C, Iversen BM. Afferent arteriopathy and glomerular collapse but not segmental sclerosis induce tubulointerstitial injury in old spontaneously hypertensive rats. Virchows Arch. 2011 Jul;459(1):99–108.
20 Alkharfi KM, Ahmed M, Yakout SM, Al-Daghri NM. Effects of calcitriol on structural changes of kidney in C57BL/6J mouse model. Int J Clin Exp Med. 2015 Aug;8(8):12390–6.
21 Rangan GK, Wang Y, Tay YC, Harris DC. Inhibition of nuclear factor-kappaB activation reduces cortical tubulointerstitial injury in proteinuric rats. Kidney Int. 1999 Jul;56(1):118–34.
22 Kang J, Dai XS, Yu TR, Wen B, Yang ZW. Glycogen accumulation in renal tubules, a key morphological change in the diabetic rat kidney. Acta Diabetol. 2005 Jun;42(2):110–6.
23 Praljak N, Ryan SD, Resnick A. Pulsatile flow through idealized renal tubules: fluid-structure interaction and dynamic pathologies. Math Biosci Eng. 2019 Dec;17(2):1787–807.
24 Weinstein AM. A mathematical model of the rat proximal tubule. Am J Physiol. 1986 May; 250(5 Pt 2):F860–73.
25 Nigro M, Viggiano D, Ragone V, Trabace T, di Palma A, Rossini M, et al. A cross-sectional study on the relationship between hematological data and quantitative morphological indices from kidney biopsies in different glomerular diseases. BMC Nephrol. 2018 Mar; 19(1):62.
26 Gil J, Gimeno M, Laborda J, Nuñalva J, Belanche I. Tangential algorithm for calculation of the fractal dimension of kidney tubuli sections. Int J Morphol. 2006;24(1):31–4.
27 Karolj A, Wu HT, Radisic M. A healthy dose of chaos: using fractal frameworks for engineering higher-fidelity biomedical systems. Biomaterials. 2019 Oct;219:113963.
28 Ortega-Martínez M, Rodriguez-Flores LE, Ancel-Arellano A, Cerda-Flores RM, de-la-Garza-González C, Ancer-Rodriguez J, et al. Analysis of cell turnover in the bronchiolar epithelium through the normal aging process. Lung. 2016 Aug;194(4):581–7.
29 Ortega-Martínez M, Gutiérrez-Dávila V, Niederhauser-García A, Cerda-Flores RM, García-Juaréz J, de-la-Garza-González C, et al. Morphometric analysis of the non-epithelial areas of mouse bronchioles through the normal aging process. Am J Transl Res. 2019 Jun; 11(6):3637–44.
30 Mandarim-de-Lacerda CA, Del-Sol M. Tips for studies with quantitative morphology (morphometry and stereology). Int J Morphol. 2017;35(4):1482–94.