UNIVERSITI PUTRA MALAYSIA

HISTOLOGICAL ASSESSMENT OF EPITHELIAL-MESENCHYMAL TRANSITION IN POLYCYSTIC KIDNEYS IN TIGERS (PANTHERA TIGRIS)

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HISTOLOGICAL ASSESSMENT OF EPITHELIAL-MESENCHYMAL TRANSITION IN POLYCYSTIC KIDNEYS IN TIGERS (*PANTHERA TIGRIS*)

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Histological Assessment of Epithelial-Mesenchymal Transition In Polycystic Kidneys In Tigers (*Panthera tigris*)" by Hennsolee Moisol @ Maisol and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 - Final Year Project.

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PENILAIAN HISTOLOGI BAGI PERALIHAN EPITELIAL-MESENKIMAL DALAM GINJAL POLIKISTIK DALAM HARIMAU (PANTHERA TIGRIS)

Oleh

Hennsolee Moisol @ Maisol

2022

Penyelia: Professor Madya Dr Annas Salleh

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Penyakit buah pinggang polikistik (PKD) adalah penyakit keturunan yang diketahui boleh mengakibatkan pembentukan sista berisi cecair dalam ginjal dan juga boleh dikaitkan dengan proses yang dikenali sebagai peralihan epitelial-mesenkimal (EMT). Walaupun sering dilaporkan pada manusia dan kucing domestik seperti baka Parsi, keadaan ini jarang dilaporkan dalam kalangan kucing liar. Kajian ini menerangkan dan menilai perubahan histologi dalam ginjal harimau (Panthera tigris) menggunakan pewarnaan rutin, histokimia khas dan imunohistokimia untuk memahami peranan EMT dalam patofisiologi penyakit ini. Sampel ginjal arkib daripada harimau yang menghidap PKD dan harimau yang tidak menghidapnya telah diproses secara rutin, dipotong, lalu diwarnai dengan haematoxylin dan eosin (HE), *Masson's trichrome*, dan imunohistokimia seperti vimentin, E-cadherin, dan β-catenin, digunakan dalam kajian ini. Slaid histologi ini telah dilihat dan dinilai untuk skor keterukan mereka menggunakan perisian ImageJ. Ginjal harimau menghidap PKD menunjukkan beberapa kerosakan melalui histopatologi seperti pelbagai sista dengan saiz yang bervariasi dan dilapisi oleh epitelium yang tipis, eosinofilik berwarna merah pudar dengan sitoplasma yang sedikit tervakuolasi dan persempadanan yang tidak jelas antara tubul. Skor keterukan dalam korteks dan medula ginjal harimau menghidap PKD adalah masing-masing (4 ± 0.31) dan (4 ± 0.47), yang secara signifikan ($p<0.05$) lebih tinggi daripada ginjal bagi harimau biasa yang tidak menunjukkan kerosakan dalam kedua-dua korteks dan medula. Bagi *Masson's trichrome* dan ketiga-tiga penanda untuk imunohistokimia, taburan data adalah di antara 50-120 piksel dalam korteks dan medula ginjal harimau menghidap PKD, yang juga secara signifikan ($p<0.05$) lebih tinggi berbanding skor bagi korteks dan medula dalam ginjal harimau biasa, yang hanya menunjukkan taburan antara 5-40

piksel. Kesimpulannya, pembentukan sista dalam ginjal harimau menghidap PKD boleh melibatkan ekspresi yang jelas untuk penanda mesenkimal daripada epitelial dan stroma dalam ginjal.

Kata kunci: *Buah pinggang polikistik; harimau; peralihan epitelial-mesenkimal*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 - Final Year Project.

HISTOLOGICAL ASSESSMENT OF EPITHELIAL-MESENCHYMAL TRANSITION IN POLYCYSTIC KIDNEYS IN TIGERS (*PANTHERA TIGRIS*)

by

Hennsolee Moisol @ Maisol

2022

Supervisor: Associate Prof. Dr Annas Salleh

Co-supervisor: Dr Mohd Akmal Mohd Noor

Polycystic kidney disease (PKD) is an inherited disease known that results in the development of fluid-filled cysts in the kidneys. This is associated with a process known as epithelial-mesenchymal transition (EMT). Despite being reported in humans and domestic cats like the Persian breed, this condition is not well-reported in wild felids. This study describes and evaluates the histological changes of kidneys of tigers (Panthera tigris) using routine, special histochemical, and immunohistochemical stains to understand the role of EMT in the pathophysiology of PKD. Archived samples of kidneys from PKD tiger and normal tiger were used in this study. They were routinely processed, sectioned, and stained with haematoxylin and eosin (HE), Masson's trichrome, Vimentin, E-cadherin, and β-catenin. These slides were viewed and were assessed for their severity score using the ImageJ software. The kidneys of PKD tigers showed several histopathological lesions like multiple cysts with various sizes lined by thinned epithelium, pale eosinophilic with slightly vacuolated cytoplasm and indistinct demarcation between the tubules. The histopathological change severity score in the renal cortex and medulla of PKD tiger was (4 ± 0.31) and (4 ± 0.47), respectively, which were significantly ($p<0.05$) higher than the control kidneys that displayed no lesion in both cortex and medulla. For Masson's trichrome and all three immunohistochemical markers, the distribution ranging between 50-120 pixels in renal cortex and medulla of PKD tigers, significantly ($p<0.05$) higher compared to the control renal cortex and medulla that showed distribution ranging between 5-40 pixels. In conclusion, development of cysts in the kidneys of PKD tigers involved significant expression of mesenchymal markers by the renal epithelia and stroma.

Keywords: *Polycystic kidney disease; tiger; epithelial-mesenchymal transit*

1.0 INTRODUCTION

Polycystic kidney disease (PKD) is an autosomal dominant hereditary disorder that causes fluid-filled cysts to develop progressively, especially in the kidneys and to a lesser extent, in the liver and pancreas (Schirrer et al., 2021). The mutation of one of the two PKD1 or PKD2 genes are involved in the autosomal-dominant polycystic kidney disease (ADPKD). In contrast, a single gene which is a prominent gene called PKHD1 (polycystic kidney and liver disease 1) found in the chromosome, is mutated in autosomal-recessive polycystic kidney disease (ARPKD). (Togawa et *al*, 2011) Several animal species, including Persian kittens (*Felis catus*), sheep lambs (*Ovis aries*), Cairn Terrier puppies, and West Highland White Terrier pups (*Canis famliliaris*), have been reported to have ARPKD. (Mayer et *al*., 2021) Although the disease has been documented in humans and many species of animals, it most commonly affects domestic cats, especially the Persian breed (Schirrer et al., 2021). In wild animals, polycystic kidney disease was previously documented in red pandas (Ailurus fulgens) and lions (Panthera leo) (Makungu et al., 2013). In general, patients will succumb to renal failure as renal parenchyma deteriorates due to slow, progressive and irreversible formation of cysts. Through abnormal secretion activities, enhanced cell proliferation, and changed cell polarity, a malfunction of primary cilia proteins and signalling cascades appear to be a key component for cystogenesis. (Mayer et *al*., 2021) These multiple cysts may be formed by the obstruction and destruction of the basement membrane in the renal tubules or the disruption of tubular epithelial cell growth. Besides, the contributing genes of this condition are the numerous germline mutations in various polycystin genes (Schirrer et al., 2021).

The formation of cysts in the kidneys in cases of PKD is associated with epithelial-mesenchymal transition (EMT) (Nakanishi et al., 2011), which is a process by which epithelial cells lose their polarity and intercellular adhesion and gain migratory and invasive properties and transition themselves into mesenchymal stem cells. To start an EMT and facilitate the ability for it to complete, a variety of different molecular pathways are activated, such as the stimulation of ECM-degrading enzymes production, the activation of transcription factors, the changes in expression of particular microRNAs, the expression of specific cell-surface proteins, and rearrangement and expression of cytoskeletal proteins. (Kalluri et *al*., 2009) The signaling pathways for EMT and the formation of cysts can be very complex, but generally, loss of E-cadherin due to is regarded as the most important event in the pathogenesis of PKD, as the initial stage in creating a polarised epithelium is the assembly of the E-cadherin/β-catenin complex, and it is crucial for maintaining an epithelial morphology. (Nakanishi et al., 2011).

PKD is not well-documented in wild animals, especially in the non-domestic wild felids and the pathophysiology of this condition in big cats also remains unclear. Thus, more specific study of this condition in wild cats may aid in understanding the histopathologic lesions of PKD in tigers as well as the pathogenesis of this disease in relation to the transition of epithelial to mesenchymal cells. This study aimed to assess the histological features of the kidneys of tigers using routine, special histochemistry, and immunohistochemistry stains to understand the role of epithelial-mesenchymal transition in the pathogenesis of PKD. The hypotheses for this study are:

H_{01}: Histological features of normal kidneys of tigers are similar to polycystic kidneys.

H_{A1}: Histological features of normal kidneys of tigers are different to polycystic kidneys.

H_{02}: The lesion scores of normal kidneys of tigers are similar to polycystic kidneys through each of the routine, special histochemistry and immunohistochemistry staining.

H_{A2}: The lesion scores of normal kidneys of tigers are different to polycystic kidneys through each of the routine, special histochemistry and immunohistochemistry staining.

2.0 LITERATURE REVIEW

2.1 What is Polycystic Kidney Disease (PKD)?

Polycystic Kidney Disease (PKD) is a hereditary disorder that gradually develops fluid-filled cysts in the kidney and, in some cases, in other organs like the liver and pancreas. (Schirrer et *al*., 2021) This hereditary disease of the autosomal-dominant PKD is brought on by a mutation in either of the two PKD1 or PKD2 genes, while the autosomal-recessive PKD is brought on by a mutation in just the one PKHD1 gene (polycystic kidney & hepatic disease 1). (Togawa et *al*., 2010) As one of the most common hereditary disorders in cats, this condition is also quite common in Persian breed cats. However, other breeds like the Exotic Shorthair, Himalayan, British Shorthair, American Shorthair, Burmilla, Ragdoll, Maine Coon, Neva Masquerade, and Chartreaux breeds may also be affected by this illness. (Schirrer et *al*., 2021)

Obstructive lesions, damage to the tubular basement membrane, or irregular tubular epithelial cell development are all potential causes of renal cysts. In addition, significant deviations from the norm in cell-matrix interactions, ion and fluid secretion, and particular membrane protein polarity might result in cyst development. (Gerhauser et *al*., 2009) The progression of these cysts may lead to irreversible kidney failure by causing the renal parenchyma to deteriorate and kidney function to gradually decline.

Since PKD appears as chronic renal failure, its clinical symptoms are not pathognomonic. Clinical symptoms such as apathy, anorexia, weight loss, an unsightly coat, polyuria, polydipsia, and gastrointestinal issues can commonly be seen.

Moreover, there is no curative treatment for PKD; only palliative medication is available to alleviate the clinical symptoms. (Schirrer et *al*., 2021)

2.2 Pathogenesis of PKD

The aetiology of the disease still has a wide range of potential causes that are being studied, and the pathogenesis pathways remain poorly understood. However, the development of the cysts may be linked to a hyperplasia of the tubular epithelium, which results in a partial obstruction of the tubules and blockage the flow of urine. The hyperplasia may be due to the alteration of polycystin-1 protein, a result of PKD1 gene mutation which then be expressed in the primary cilium, a flagellar structure arising from the tubular cell and in contact with the urinary flow. (Schirrer et *al*., 2021) The primary cilia control the proliferation, polarisation, and differentiation of the renal tubular epithelial cells using calcium-dependent second messenger signalling pathways, such as Ras or mitogen-activated protein kinases. The term "ciliopathies" refers to conditions that cause abnormalities in this complex, where a malfunction of primary cilia proteins and signalling pathways appears to be a key component for cystogenesis through abnormal secretion activities, enhanced cell proliferation, and changed cell polarity. (Mayer et *al*., 2021) As a result, the loss of cilia polarisation would affect the function of water absorption which will lead to the development of cysts in the kidney parenchyma. Clinical examination may reveal general dehydration, pale mucous membranes, increased volume, and on kidneys palpation, an abnormal kidney contour can be identified. (Schirrer et *al*., 2021)

The development of cysts in the parenchyma appears to be a result of a process that involves enhanced cell proliferation, fluid secretion, and changes to the extracellular matrix. In relation to an aberrant extracellular matrix, renal interstitial fibrosis is a significant feature in PKD and the epithelial-to-mesenchymal transition (EMT) is among the hallmarks of fibrosis. (Togawa et *al*., 2010)

In addition to its well-known antiapoptotic function, mutant polycystin-1 appears to be important for tubular epithelial cell proliferation and differentiation in cats. Thus, a crucial component in the development of cysts is the fine balance between tubular degradation, necrosis activation, and apoptosis. (Schirrer et *al*., 2021)

2.3 Epithelial to mesenchymal transition

An epithelial-mesenchymal transition (EMT) is a biological process that enables polarised epithelial cells to go through a number of biochemical changes that allow them to adopt a mesenchymal cell phenotype, which includes increased invasiveness, migratory capacity, resistance to apoptosis, and production of extracellular matrix components. (Kalluri et *al*., 2009) This transition where the epithelial cells acquire mesenchymal properties, is one of the main characteristics in the renal interstitial fibrosis which is also one of the crucial features in PKD. There are various markers that suggest a correlation between the primary cilium and renal cystic diseases, which are associated with a group of conditions known as ciliopathies that include abnormalities in the primary cilium. However, the process of EMT of tubular epithelia which can be caused by the ciliopathy gene product limitations is yet to be unclear. (Togawa et *al*., 2010)

Compromised cell polarity and epithelio-mesenchymal transition (EMT) are key components of cystogenesis and pathogenesis, respectively, in cystic nephropathies, through immunohistochemical findings. The process of EMT, which is characterised by a transiently altered expression pattern of mesenchymal (vimentin) and epithelial (cytokeratin) marker proteins as well as the downregulation of cytoskeletal components and adhesion proteins like E-cadherin, requires the loss of the apico-basal cell polarisation. (Mayer et *al*., 2021) The production of a polarised epithelium begins with the assembly of the E-cadherin/β-catenin complex, which is crucial for maintaining an epithelial phenotype. Hence, abnormal expression of E-cadherin and/or β-catenin may be related to PKD, while loss of E-cadherin expression is a distinctive feature of EMT. Loss of E-cadherin expression is closely linked with plasma membrane instability, which is seen during foetal development as well as in several pathological processes such carcinogenesis, cancer cell metastasis, and EMT. (Togawa et *al*., 2010)

Concepts like EMT and transdifferentiation were first used in oncology and embryology, respectively. A well-known process that defines embryonal plasticity is the cell switching between epithelial and mesenchymal phenotype by turning on and off particular genes throughout development. (Chea et *al*., 2009) The occurrence of the opposite process, a mesenchymal-epithelial transition (MET), which includes the conversion of mesenchymal cells into epithelial derivatives, reveals the phenotypic plasticity provided by an EMT. (Kalluri et *al*., 2009)

2.4 Renal diseases in non-domesticated felids

Chronic kidney disease (CKD), a prevalent disease involving geriatric Felidae, is characterised as the presence of structural or functional abnormalities in one or both kidneys. The pathophysiology of feline CKD is complex and involves particular disease processes, such as polycystic kidney disease, renal amyloidosis, renal dysplasia, and renal lymphoma, which result in renal damage or malfunction. (Lawson et *al*., 2014) Despite having a heterogenous aetiology, the majority of cases include non-specific renal lesions, such as fibrosis and chronic tubulointerstitial inflammation. (S.W. Lee et *al*., 2018) Studies also shows that tubulointerstitial nephritis is the most prevalent kidney pathology in exotic felids, including tigers, lions, cougars, leopards, snow leopards, clouded leopards, Canadian lynx, ocelots, bobcats, cheetahs, and jaguars. (Newkirk et *al*., 2010)

Chronic kidney disease in exotic felids was known to be linked with amyloidosis, glomerulonephritis, glomerulosclerosis, interstitial nephritis, and pyelitis. Renal medullary amyloidosis may also induce a constriction on the medullary blood vessels, which can lead to renal papillary necrosis. (Newkirk et *al*., 2010) Necrosis of the inner medulla, a defining characteristic of renal papillary necrosis, is linked to coagulative necrosis of the renal papillae and medullary pyramids. (S.W. Lee et *al*., 2018)

Typical diagnoses for tubulointerstitial nephritis include hematogenous infectious agents, however it is also possible for it to develop as a result of ascending pyelonephritis, primary glomerular disease, or a previous occurrence of acute renal failure. While in the case of pyelitis, it may have been caused by chronic or prior

episodes of pyelonephritis, similar to tubulointerstitial nephritis. (Newkirk et *al*., 2010)

Nevertheless, irrespective of the underlying etiology, the progressive feature of renal fibrosis leads to a decline in renal function regardless of the original renal injury. (Lawson et *al*., 2015)

2.5 What are non-domesticated felids?

There are at least 36 species of non-domesticated cats in the family Felidae. Felid taxonomy has undergone extensive study, and at least 12 genera are now recognised as a result of numerous morphological and genetics research. They range in size from 1 kilogramme (kg) to 300 kg, making them the existing carnivore family with the broadest variety of body sizes. (Lamberski, 2014)

All continents, with the exception of Australasia and Antarctica, are home to the wild Felidae, which are also found on several islands, both huge (Borneo) and small (Trinidad). (Macdonald et *al*., 2010) Predators like wild felids need a significant amount of space and a good density of prey. Both of these requirements have been adversely impacted by the increasing human population, which has led to a widespread reduction in the range and population of all felid species. (Lamberski, 2014)

The anatomy of the majority of felids is extremely similar, which is relevant with their shared responsibilities of capturing, subduing, and consuming their prey. (Macdonald et *al*., 2010) Due to their unwavering commitment to devouring flesh, often that of vertebrate prey, felids are at the extreme of the carnivore food chain. More recent studies also discovered that the presence of long, fleshy, elastic vocal

folds within the larynx of big cats—which vibrate to produce a roar—was the primary distinction between the roaring cats and the others. (Lamberski, 2014)

3.0 MATERIALS AND METHODS

3.1 SAMPLE

For this study, previously collected samples of the kidney of tiger (*Panthera tigris*) were used. Two paraffin-embedded normal kidney samples and two polycystic kidney samples of tiger were retrieved from Histopathology Laboratory, Faculty of Veterinary Medicine, University Putra Malaysia. These tissues were routinely sectioned and stained with routine stain (hematoxylin and eosin / HE), special histochemical stain (Masson's trichrome), and immunohistochemical stains (E-cadherin, β-catenin, and vimentin) which are important for evaluation of epithelial-mesenchymal transition.

3.2 EXAMINATION OF HISTOLOGY SLIDES

The general features of the normal and polycystic kidneys can be described through the evaluation of the slides stained with HE. The tissue section on slides can be evaluated through a severity scoring based on the distribution of lesion which was slightly modified from Hoshino et *al*., 2015. There are five sets of scores, 0 for no lesion, 1 for 1-10% lesion, 2 for 11-20% lesion, 3 for 21-30% lesion, and 4 for lesions that are more than 30%. The evaluation was done in any 10 random high power fields of the cortex and also the medulla from each of the kidney samples. The distribution of lesions was calculated by dividing the total of affected tubules with the total of tubules in one random high power field area. For HE stain, the lesions in each tubules were described and assessed based on Mayer et *al*., 2021.

Then, for the slides stained with immunohistochemical, the evaluation was done based on the distribution of immunoreactivity of the different proteins, where the distribution of the immunohistochemical stain can be described as brown in colour.

For Masson's trichrome staining, the tissue was also evaluated based on the staining distribution. This staining can be described as bluish/greenish in colour. The scoring for both Masson's trichrome and immunohistological slides, will be done through ImageJ software Version 1.53e, where a number of colour pixels in the staining will be counted.

3.3 STATISTICAL ANALYSIS

The data of lesion severity scores were recorded and compared between the groups of control sample and PKD sample. Statistical data were then expressed in mean ± standard deviation (SD) and the difference between the two comparing groups will be analyzed using Mann-Whitney U Test with significance difference at $p<0.05$.

4.0 RESULTS

In general, all micrographs had shown a consistent result throughout the assessment, where the PKD samples showed higher scores and intensity, while the control samples showed lower scores and intensity.

4.1 ROUTINE HISTOPATHOLOGY

Histological Description

Histopathological observation on the control kidney samples showed minimal lesions and no suggestive lesions of PKD in the kidney parenchyma. The renal tubules were lined by simple cuboidal epithelium with no vacuolation in the cytoplasm. The glomeruli also appeared to be normal and the Bowman's capsule was observed to be lined by simple squamous epithelium. There is also a distinct demarcation between cells and there are no cysts present in the renal parenchyma. However, renal epithelial cells desquamation can also be observed in the control sample of the kidney. Meanwhile, for kidney samples with PKD, multiple cysts with various sizes can be observed lined by thinned epithelial cells in tissue samples. The cytoplasm of the epithelium also appeared to be pale eosinophilic and slightly vacuolated with unclear borders between the epithelial cells. Figure 1 shows the described histopathological changes.

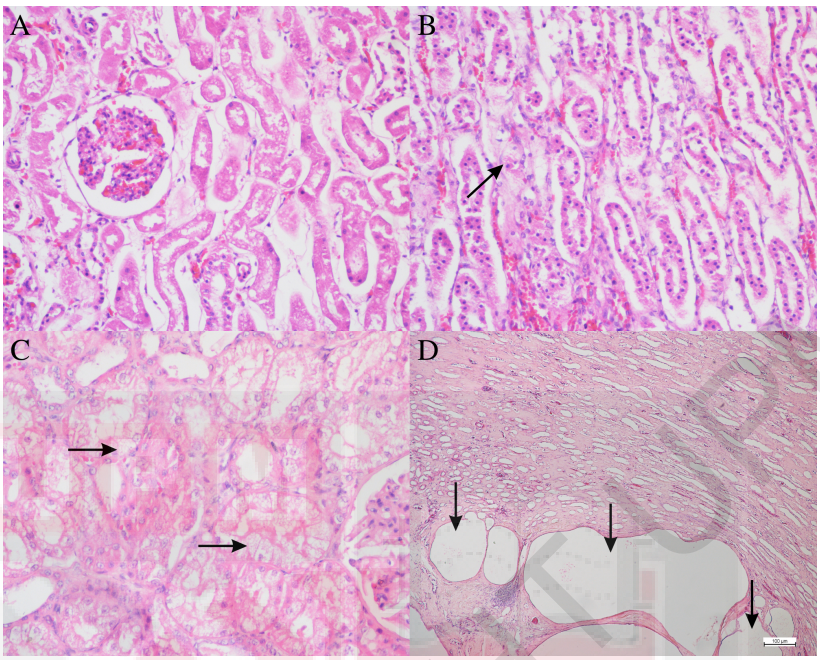

Figure 1. Routine histopathology of a control kidney parenchyma of tigers (A). Desquamation of renal epithelial cells in control kidney sample (black arrow)(B). Vacuolation in the cytoplasm of renal tubular epithelium in PKD sample (black arrow)(C). Multiple cysts with various sizes in PKD sample (black arrow)(D).

Histological Assessment

The evaluation of the renal parenchyma samples showed that the mean scores for both control and PKD sample in the cortex area were score (0.15 ± 0.37) and (3.9 ± 0.31), respectively, which indicates there was no or minimal lesion observed in control sample and more prominent lesions in the PKD sample. Similarly in the medullary area, the evaluation mean scores showed (0.05 ± 0.22) in the control sample and (3.7 ± 0.47) in the PKD sample which also indicates that the lesions in the PKD sample are more significant compared to the control sample. However, some of the areas in the control samples that were selected for evaluation also showed score 1, which indicates that there are 1-10% lesions present in the samples. For the kidney samples with PKD, it showed a consistent result where the mean scores in the cortex were score 4 which stipulates that there are more than 30% of lesions and some areas with score 3, 21-30% lesions covering the selected areas in the tissue samples (Table 1).

Sample	Cortex				Medulla			
	Control		PKD		Control		PKD	
	A	B	A	B	A	B	A	B
1	0	0	4	4	0	0	4	4
2	1	0	3	4	0	0	4	3
3	1	0	3	4	0	0	4	3
4	0	0	4	4	0	0	4	4
5	0	0	4	4	0	0	4	4
6	0	0	4	4	0	0	4	3
7	0	0	4	4	0	0	4	3
8	0	1	4	4	0	0	4	4
9	0	0	4	4	0	1	3	3
10	0	0	4	4	0	0	4	4
Mean	0.15		3.90		0.05		3.70	
SD	0.37		0.31		0.22		0.47	

Table 1. Summary of the severity score of the renal parenchyma in cortex and medulla of the control and PKD sample of tiger's kidneys.

4.2 MASSON'S TRICHROME

Histological Description

The control kidney sample showed an inconspicuous expression of the bluish Masson's trichrome stains in the micrographs. There is minimal or nearly absent scarring in both cortical and medullary areas of the renal parenchyma in the control sample. The demarcation between tubules were prominent and there is also no tubular atrophy identified in the sample. Intact glomeruli can also be identified and no cytoplasmic vacuolation in the epithelial cells observed. In contrast, the kidney samples of PKD tiger showed a very prominent bluish Masson's trichrome expression particularly in the renal tubulointerstitial. Interstitial fibrosis also appeared to be covering at least half of the micrographs and also a very prominent cortical and medullary scarring can be observed. Glomerulosclerosis was observed in the cortex area of the sample and tubular atrophy in both cortex and medulla as well as cytoplasmic vacuolization of the epithelium can also be seen in the renal parenchyma (Figure 2).

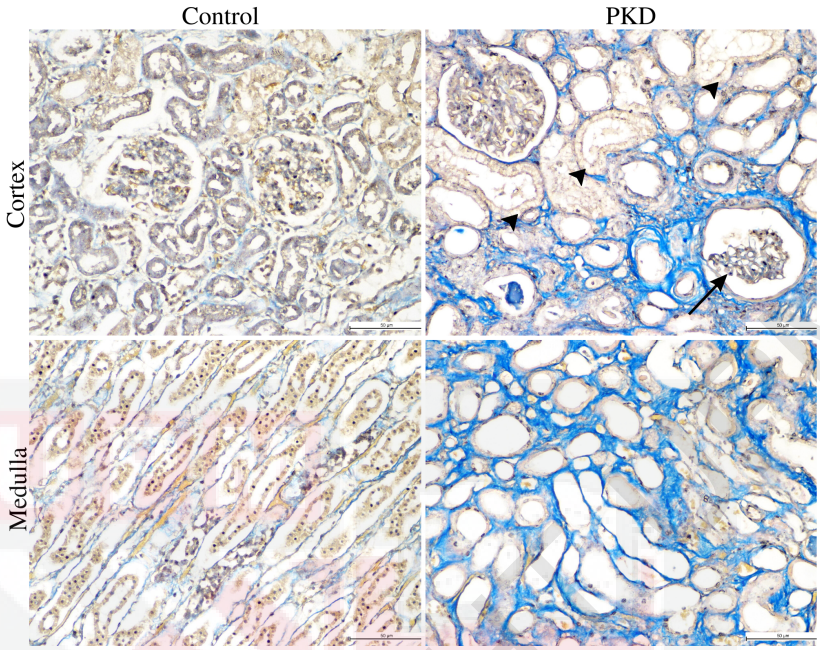

Figure 2. Masson's trichrome staining on negative control and PKD sample of tiger's kidney. Glomerulosclerosis on the cortical region of the PKD sample (black arrow). Vacuolization in the cytoplasm of the renal epithelial cells of PKD sample (black arrowhead).

Histological Assessment

The evaluation of kidney tissue samples stained with Masson's trichrome showed that the mean distributions of the stain in cortical region, for both control and PKD samples were (11.4 ± 9.9) and (69.5 ± 23.7), respectively. Hence, more prominent Masson's trichrome staining distributions in the PKD sample compared to the control sample can be observed. In the medullary region, the mean distribution of the staining in control and PKD sample were (19.8 ± 13.9) and (80.1 ± 20.7),

respectively, which also indicate that the staining distribution in PKD sample was more notable compared to the control sample (Table 2).

Sample	Cortex				Medulla			
	Control		PKD		Control		PKD	
	A	B	A	B	A	B	A	B
1	1	2	35	62	2	26	55	93
2	3	10	40	88	16	20	69	106
3	6	37	34	114	0	46	65	97
4	22	19	43	70	3	0	64	86
5	1	22	51	69	9	30	69	87
6	11	19	44	86	10	35	72	94
7	16	0	67	92	29	33	57	124
8	0	11	61	76	18	39	69	83
9	8	18	77	113	4	25	52	104
10	3	19	86	82	20	30	52	104
Mean	11.4		69.5		19.8		80.1	
SD	9.9		23.7		13.9		20.7	

Table 2. Summary of the Masson's trichrome distribution in cortex and medulla of the control and PKD sample of tiger's kidneys.

4.3 IMMUNOHISTOCHEMISTRY

Histological Description

There was a nearly absent expression of brownish vimentin in the cortical area of the control sample. As for the medullary region of the control sample, mild expression of vimentin can be observed particularly in the tubulointerstitial area. Meanwhile in the PKD sample, a very significant vimentin expression was observed in the cortical area as well as in the medullary region. The expression of vimentin in the renal parenchyma of PKD sample was more intense especially in the tubulointerstitial area. The demarcation between the renal tubules was also not clear in the PKD sample compared to the control sample (Figure 3).

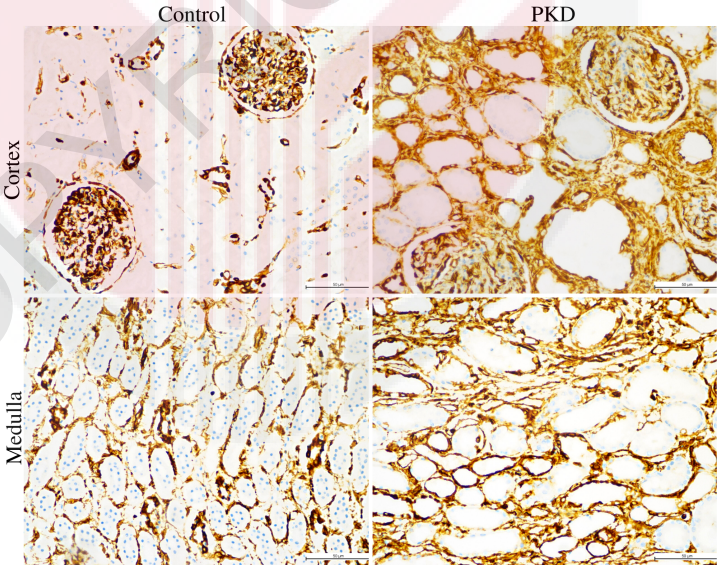

Figure 3. Expression of vimentin both in cortex and medulla of control and PKD sample through immunohistochemistry staining.

The control kidney sample displayed copious expression of E-cadherin in the medullary region compared to the cortex. The brownish immunostaining of E-

cadherin in the cortical area of the control sample was slight particularly in the basolateral and cytoplasm part of the tubular epithelial cells. There were no expressions visible in the glomeruli of the cortical area and the epithelium lining the tubules appeared to be well intact in the control sample. However, kidney samples of tigers with PKD showed a very notable expression of E-cadherin in the tubular epithelium especially in the basolateral area as well as the cytoplasm. The distribution of immunostaining in the PKD sample was equivalent to the control sample as there were significant expressions of the immunostaining that can be observed in the medullary region compared to the cortex area. Besides, the glomeruli in the PKD samples showed mild expressions and the cytoplasm of the tubular epithelium also appeared to be slightly foamy (Figure 4).

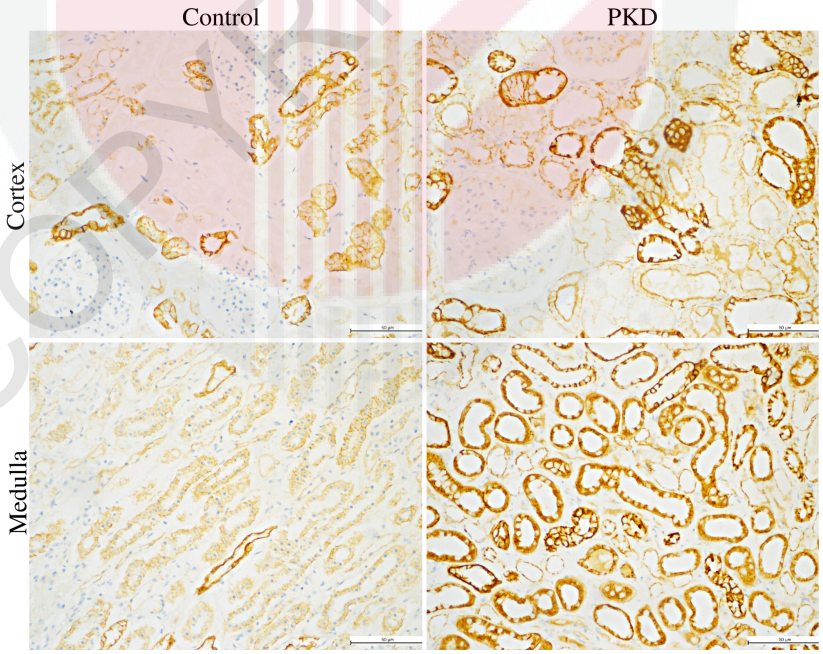

Figure 4. Expression of E-cadherin both in cortex and medulla of control and PKD sample through immunohistochemistry staining.

As for β-catenin, milder expression of the immunostaining in the basolateral area of the renal tubular epithelium can be observed in the control sample both in cortical and medullary regions. Apart from that, the renal tubular epithelial cells also appeared to be well intact and the borders between tubules were also distinct. Meanwhile for the PKD sample, the expression of the β-catenin was very significant especially in the cytoplasm of the tubular epithelial cells and moderate expression in the nuclei part of the cells. The cytoplasm of the tubular epithelium was slightly vacuolated and tubular atrophy can be observed in the micrographs (Figure 5).

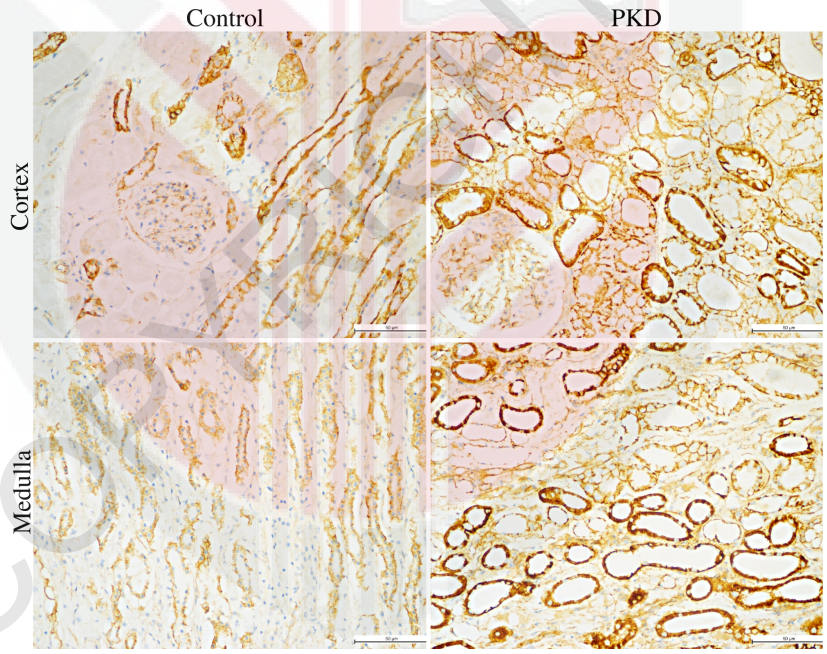

Figure 5. Expression of β-catenin both in cortex and medulla of control and PKD sample through immunohistochemistry staining.

Histological Assessment

The kidney tissue sample stained with vimentin showed that the mean distribution of the immunostaining through the assessment in the cortical region, both

in negative control and PKD sample were (32.3 ± 25.0) and (93.8 ± 28.5), respectively. Meanwhile, for the medullary region, the control sample showed mean distribution of (38.4 ± 25.2), while the PKD sample showed mean distribution of (98.1 ± 12.3).

The evaluation of kidney parenchyma samples stained with E-cadherin showed that the mean distribution of the stains in the cortex, both in control and PKD samples were (19.4 ± 17.4) and (66.0 ± 22.9), respectively. Moreover, in the medulla, the mean distribution of the immunostaining in the negative control sample was (9.3 ± 8.3) and in the PKD sample was (65.1 ± 10.9).

As for β-catenin, the mean distribution of the immunostaining in the cortical area of the negative control kidney tissue sample was (29.5 ± 16.6), while in the PKD sample was (78.7 ± 17.7). Furthermore, in the medullary region, the mean distribution of the stain both in negative control and PKD sample were (9.0 ± 8.2) and (74.9 ± 18.4), respectively. Table 3 summarizes the scoring for the expression of these markers in the kidneys of tiger with and without PKD.

Sample		Cortex				Medulla			
		Control		PKD		Control		PKD	
		A	B	A	B	A	B	A	B
Vimentin	1	81	10	53	108	79	9	93	91
	2	17	0	72	120	60	31	94	96
	3	28	48	77	129	33	7	96	114
	4	45	14	73	116	20	45	89	90
	5	15	79	56	124	57	65	103	123
	6	57	4	53	128	37	62	94	114
	7	17	43	84	109	24	0	99	116
	8	19	16	67	102	23	82	82	94
	9	0	51	65	140	37	65	95	112
	10	42	60	81	118	0	31	73	94
	Mean	32.3		93.8		38.4		98.1	
	SD	25.0		28.5		25.2		12.3	

Table 3. Summary of the vimentin distribution in cortex and medulla of the control and PKD sample of tiger's kidneys.

Sample		Cortex				Medulla			
		Control		PKD		Control		PKD	
		A	B	A	B	A	B	A	B
E-cadherin	1	9	35	46	89	10	10	55	59
	2	8	1	65	87	0	0	66	46
	3	21	4	59	36	6	8	73	55
	4	0	6	55	48	0	9	68	84
	5	14	37	32	86	14	17	58	74
	6	11	0	56	94	11	11	60	46
	7	1	51	27	98	6	8	58	79
	8	13	44	44	82	7	6	59	70
	9	18	49	60	94	8	39	64	78
	10	24	41	72	90	3	12	70	79
	Mean	19.4		66.0		9.3		65.1	
	SD	17.4		22.9		8.3		10.9	

Table 4. Summary of the E-cadherin distribution in cortex and medulla of the control and PKD sample of tiger's kidneys.

Sample		Cortex				Medulla			
		Control		PKD		Control		PKD	
		A	B	A	B	A	B	A	B
β-catenin	1	20	0	57	93	7	23	78	73
	2	50	49	66	67	0	6	65	32
	3	10	48	75	91	5	20	78	101
	4	49	12	64	67	8	3	83	82
	5	0	41	64	105	17	27	25	90
	6	32	45	58	90	2	0	67	72
	7	21	21	82	98	8	3	74	98
	8	31	18	61	107	18	4	75	86
	9	29	46	62	99	10	0	70	79
	10	23	44	64	103	15	3	87	82
	Mean	29.5		78.7		9.0		74.9	
	SD	16.6		17.7		8.2		18.4	

Table 5. Summary of the β-catenin distribution in cortex and medulla of the control and PKD sample of tiger's kidneys.

5.0 DISCUSSION

Routine staining (Hematoxylin & eosin) was used in this study in order to observe and describe the general features of both the negative control and PKD samples. Based on a study done by Gerhauser et *al*., 2008, multiple cysts were seen in the kidney histology of a lion with PKD, which were lined by a flattened or cuboidal single-layered epithelium, which is also similar with the histological findings in this study as well as several other species, such as goats (Mayer et *al*., 2021), domesticated feline (Bosje et *al*., 1998), and also rats (Togawa et *al*., 2011). According to the routine histopathology findings, the renal tubular epithelium showed a progressive deterioration of the functional parenchyma through the development of the cysts. The continuation of this renal parenchyma degradation may lead to irreversible kidney damage. In addition, desquamation of the renal epithelium was also observed in the negative control kidney sample, which may be caused by an error in sampling or loss of cellular architecture due to post mortem changes throughout the preparation of samples. However, it is not a suggestive lesion of PKD in tigers.

Special histochemistry which is Masson's trichrome was used in this study, as this staining is known to detect the presence of delicate fibrillary collagenous fibers in the parenchyma which may facilitate the understanding of the transition of epithelial to mesenchymal cells. The tiger's kidney sample with PKD showed more prominent bluish Masson's trichrome expression especially in the tubulointerstitial area compared to the control sample. This may indicate that there is a higher amount of delicate fibrillary collagenous fibers in that area, which may be caused by tubulointerstitial fibrosis. Hence, the transition of epithelial to mesenchymal transition

can be suggested in this renal parenchyma, as the epithelial-mesenchymal transition is one of the key features in the formation of fibrosis (Mayer et *al*., 2021).

In this study, immunohistochemistry was used in detecting the presence of three different types of protein, vimentin, E-cadherin, and β-catenin, which may assist in the understanding of the pathophysiology of PKD in tigers, in relation to the deterioration of cell polarity and EMT. Vimentin expressions were assessed as a mesenchymal marker, and were used to detect the presence of mesenchymal cells which may be crucial in the transition of epithelial cells into mesenchymal cells. The PKD sample showed a very significant expression of vimentin especially in the tubulointerstitial region, which may suggest the accumulation of fibroblasts. Vimentin expression was elevated in the interstitial fibrotic regions adjacent to giant cysts, indicating an abundance of fibroblasts (Togawa et *al*., 2011). E-cadherin and β-catenin complex is very important in the cell to cell adhesion as well as in maintaining the features of an epithelial cell. Hence, normal kidney parenchyma may show a higher expression of these proteins and should be reduced in the tiger's kidney with PKD. According to previous studies, E-cadherin loss is one of the characteristics of EMT, and abnormal E-cadherin and/or β-catenin expression may be related to PKD. However, in the recent study, the expression of both E-cadherin and β-catenin were relatively higher in the PKD sample compared to the negative control sample. This condition may be due to the random area selection for the histological assessment, which may suggest that a non-cystic area might be selected for the examination. Based on a study done by Togawa et *al*., 2011, the expression of E-cadherin in non-cystic tubules will remain high even in the tiger's kidney with PKD.

Since PKD is well-known to be an inherited disease that can pass down the responsible mutated gene to the next generation, it is very crucial to detect the affected animals in an early stage to allow the introduction of selection strategies of healthy animals in their breeding lines (Schirrer et *al*., 2021). The implementation of these selection strategies may reduce the prevalence of this condition in animals, where in this case, it might also improve the conservation plans for non-domestic felines. Hence, in order to eradicate this condition in the breeding line of an animal species, it is crucial to understand multiple methods of diagnosis in facilitating the process of diagnosing the diseased animals. According to this study, the histopathology results showed that there is a presence of lesions which may suggest PKD as well as the presence of the transition of epithelial to mesenchymal cells, which also can indicate this condition in the animals. However, histopathology findings alone may not be adequate to determine whether this lesion indicated as an early PKD lesion or an accidental finding, as some nephropathy in animals may share the same histopathological result with PKD (Helps et *al*., 2007). Diagnostic imaging is commonly used to make an ADPKD diagnosis. Renal ultrasound is frequently utilized since it is inexpensive, safe, as well as has a high level of dependability (Perrone et *al*., 2014). The cysts may appear as hypo- to anechoic spherical cavities that range in size from one to more than twenty millimetres and may be connected to a subsequent contrast. Developed genetic study methods were recently being used in diagnosing PKD and various techniques have been developed to detect the mutation that causes the disease, based on the discovery of the gene involved in the development of PKD (Schirrer et *al*., 2021). However, the results of the genetic analysis in a current study showed no correlation of cyst formation indicated by ultrasonography. Previous

research demonstrated that some cats were diagnosed with renal cysts through ultrasonography but showed a negative test result for the PKD1 mutation even if a cyst-like structure was observed through the ultrasound. Despite the fact that renal cysts in PKD are easily identified by ultrasonography, hydronephrosis with a dilated renal pelvis or renal cysts created by dilation of urinary tubules in late stage chronic nephritis might also be misinterpreted as PKD cysts (Sato et *al*., 2019). Although molecular tests can be used to diagnose PKD because the pathology is caused by a point mutation, their limited availability and high cost may prevent them from being used in daily clinical settings. Instead, ultrasound has proven to be the most effective imaging modality for evaluating renal phenotypic in potentially affected animals (Schirrer et *al*., 2021).

All data were analyzed using a Statistical Analysis Software, SPSS under a non-parametric Mann Whitney U test. The results show that the p value is less than 0.05, which indicates that there is a statistically significant difference between the groups. Hence, null hypotheses are rejected.

6.0 CONCLUSION

In conclusion, the PKD sample is different compared to the negative control sample of the kidney of tigers, in terms of their histological features as well as the lesion scores through each of the routine, special histochemistry and immunochemistry staining. Furthermore, the renal epithelial cells of PKD sample in this study also displayed mesenchymal cells characteristics which may suggest the presence of the transition of the epithelial cells into mesenchymal cells. Besides, the immunohistochemistry results also showed the involvement of the transition in the formation of the cysts in the renal parenchyma. Hence, the EMT plays an important role in the pathogenesis of PKD in tigers. For future studies, the evaluation of the renal epithelial cells should be conducted based on the types of renal tubules, as the expression of some proteins in immunohistochemistry can be affected by the types of tubules.

7.0 REFERENCES

Beck, C., & Lavelle, R. B. (2001). Feline polycystic kidney disease in Persian and other cats: A prospective study using ultrasonography. Australian Veterinary Journal, 79(3), 181–184.

Biller, D. S., DiBartola, S. P., Eaton, K. A., Pflueger, S., Wellman, M. L., & Radin, M. J. (1996). Inheritance of polycystic kidney disease in Persian Cats. Journal of Heredity, 87(1), 1–5.

Bosje, J. T., & van den Ingh, T. S. G. A. M. (1998). Polycystic kidney disease in cats. Veterinary Quarterly, 20(sup1).

Chea, S. W., & Lee, K. B. (n.d.). *TGF-β mediated epithelial-mesenchymal transition in autosomal dominant ...* TGF-β Mediated Epithelial-Mesenchymal Transition in Autosomal Dominant Polycystic Kidney Disease. Retrieved September 23, 2022, from https://www.researchgate.net/publication/24175493_TGF-b_Mediated_Epithelial-Mesenchymal_Transition_in_Autosomal_Dominant_Polycystic_Kidney_Disease/fulltext/0f612d5e3829848d99d11db0/24175493_TGF-b_Mediated_Epithelial-Mesenchymal_Transition_in_Autosomal_Dominant_Polycystic_Kidney_Disease.pdf

Gerhauser, I., Philipp, U., Distl, O., & Beineke, A. (2009, August). Multiple cyst formation in the liver and kidneys of a lion (Panthera leo). Retrieved December

5, 2022, from https://www.researchgate.net/publication/226272393_Multiple_cyst_formation_in_the_liver_and_kidneys_of_a_lion_Panthera_leo

Helps, C., Tasker, S., & Harley, R. (2007). Correlation of the feline PKD1 genetic mutation with cases of PKD diagnosed by pathological examination. Experimental and Molecular Pathology, 83(2), 264–268.

Hoshino, J., Mise, K., Ueno, T., Imafuku, A., Kawada, M., Sumida, K., Hiramatsu, R., Hasegawa, E., Yamanouchi, M., Hayami, N., Suwabe, T., Sawa, N., Hara, S., Fujii, T., Ohashi, K., Ubara, Y., & Takaichi, K. (2015, June 11). A pathological scoring system to predict renal outcome in diabetic nephropathy. American Journal of Nephrology. Retrieved October 18, 2022, from https://www.karger.com/Article/Fulltext/431333

Junginger, J., Hansmann, F., Herder, V., Lehmbecker, A., Peters, M., Beyerbach, M., Wohlsein, P., & Baumgärtner, W. (2015). Pathology in captive wild felids at German Zoological Gardens. PLOS ONE, 10(6).

Kalluri, R., & Weinberg, R. A. (2009, June). *The basics of epithelial-mesenchymal transition*. The Journal of clinical investigation. Retrieved September 23, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2689101/

Lamberski, N. (2014, June 25). *Felidae*. Fowler's Zoo and Wild Animal Medicine, Volume 8. Retrieved September 23, 2022, from https://www.sciencedirect.com/science/article/pii/B9781455773978000475?via%3Dihub

Lawson, J., Elliott, J., Wheeler-Jones, C., Syme, H., & Jepson, R. (2014, October 24). *Renal fibrosis in feline chronic kidney disease: Known mediators and mechanisms of injury*. The Veterinary Journal. Retrieved September 23, 2022, from https://www.sciencedirect.com/science/article/pii/S1090023314004225?via%3Dihub#bib0555

Lee, S. W., Elfadl, A. K., Chung, M. J., Ullah, H. M. A., Yuh, D. K., Lee, S. H., Jeong, K. S., & Park, J. K. (2018, October 16). *Urocystitis, Pyelonephritis, renal papillary necrosis and chronic tubulointerstitial disease causing chronic renal insufficiency in a Siberian tiger (Panthera tigris altaica): A case report*. Veterinární medicína. Retrieved September 23, 2022, from https://www.agriculturejournals.cz/web/vetmed.htm?type=article&id=132_2017-VETMED

Lee, Y. J., Chen, H. Y., Hsu, W. L., Ou, C. M., & Wong, M. L. (2010). Diagnosis of feline polycystic kidney disease by a combination of ultrasonographic examination and PKD1 gene analysis. Veterinary Record, 167(16), 614–618.

Macdonald, D. W., & Loveridge, A. J. (2011). Biology and conservation of wild felids. Oxford University Press.

Makungu, M., Plessis, W., Barrows, M., & Koeppel, K. (2013, September). Polycystic kidneys in the red panda (ailurus fulgens). Retrieved December 5, 2022, from

https://www.researchgate.net/publication/257029036_Polycystic_kidneys_in_the_red_panda_Ailurus_Fulgens

Mayer, C., Ormanns, S., & Majzoub-Altweck, M. (2021, January 19). *Histomorphology and immunohistochemistry of a congenital nephromegaly demonstrate concurrent features of heritable and acquired Cystic Nephropathies in a girgentana goat (Capra Falconeri)*. Case reports in veterinary medicine. Retrieved September 23, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7837792/

McConnachie, D. J., Stow, J. L., & Mallett, A. J. (2021). Ciliopathies and the kidney: A Review. American Journal of Kidney Diseases, 77(3), 410–419.

Newkirk, K. M., Newman, S. J., White, L. A., Rohrbach, B. W., & Ramsay, E. C. (2010, September 28). *(PDF) renal lesions of nondomestic felids - researchgate*. Renal Lesions of Nondomestic Felids. Retrieved September 23, 2022, from https://www.researchgate.net/publication/46579032_Renal_Lesions_of_Nondomestic_Felids

Sato, R., Uchida, N., Kawana, Y., Tozuka, M., Kobayashi, S., Hanyu, N., Konno, Y., Iguchi, A., Yamasaki, Y., Kuramochi, K., & Yamasaki, M. (2019). Epidemiological evaluation of cats associated with feline polycystic kidney disease caused by the feline PKD1 genetic mutation in Japan. Journal of Veterinary Medical Science, 81(7), 1006–1011.

Schirrer, L., Marín-García, P. J., & Llobat, L. (2021, November 8). *Feline polycystic kidney disease: An update*. MDPI. Retrieved September 23, 2022, from https://www.mdpi.com/2306-7381/8/11/269/htm

Togawa, H., Nakanishi, K., Mukaiyama, H., Hama, T., Shima, Y., Sako, M., Miyajima, M., Nozu, K., Nishii, K., Nagao, S., Takahashi, H., Iijima, K., & Yoshikawa, N. (2011). Epithelial-to-mesenchymal transition in cyst lining epithelial cells in an orthologous PCK rat model of autosomal-recessive polycystic kidney disease. American Journal of Physiology-Renal Physiology, 300(2).