nature portfolio

Peer Review File

Multiscale and Multimodal Evaluation of Autosomal Dominant Polycystic Kidney Disease Development

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This study performs a detailed analysis of Pkd2 cko mice induced for Pkd2 loss starting at 4 wks. The animals are analyzed histologically, for survival, and by MR and CT imaging. The study provides quite good analysis of the progression in this model.

Specific points:
1. The animal numbers per group are very small 3 per sex for many endpoints, making it difficult to be precise about the course of disease development. This especially true because of variably between animals.
2. Automated methods for determining TKV have been described by only semiautomated methods are described here.
3. The accuracy of the CT masks to determine cystic regions is uncertain as these volumes seem much more variable than the TKV measurements.
4. It is not clear why animals with more severe disease, 12 wks and later after induction, were not imaged.
5. It is unclear why when using a 7-Tesla Biospec 70/20 scanner that the resolution is too low to measure TKV?
6. The model develops very quickly between 8 wks and 12 wks after induction. Were their efforts to analysis this period in more detail?
7. Was Pkd2 expression monitored within the flox flanked deleted region? It would be more helpful to analyze PC2 protein level in the cko kidneys.

Reviewer #2 (Remarks to the Author):

This is a very interesting manuscript that presents a workflow for assessing the progression of cystic regions in animal models of PKD using histology, CT and MRI.

The authors use the term "coalescent" and its meaning is unclear.
->Does this term mean that the cysts appear to "fuse" into a region because the distance between the cysts is below the resolution limit of the technique?

->Is cyst fusion something that is described or seen in histology of PKD?

->Can these methods used distinguish between normal interstitium and fibrosis?

-->Can these methods distinguish or detect calcifications such as kidney stone disease or Randall's plaques?

Reviewer #3 (Remarks to the Author):

Dear Editor,

I am writing in response to the manuscript titled " Multiscale and Multimodal Evaluation of Autosomal Dominant Polycystic Kidney Disease Development" by Pablo Delgado-Rodriguez, Nicol´as Lamanna-Rama, Cassondra Saande , Rafael Aldabe , Mar´ıa Luisa Soto-Montenegro , Arrate Munoz-Barrutia.

I would like to commend the authors for their insightful study. The study workflow represents a comprehensive approach for assessing the progression of PKD using CT, MRI, and histology, and the simultaneous utilization of these modalities is quite unique. However, I have some concerns and additional points that I believe could contribute to the discussion.

Firstly, I noticed that the use of a contrast agent in the CT evaluation of the kidney parenchyma was not mentioned in the Materials and Methods section. In Figure 6, a contrast agent appears to be present in the collective system of the kidneys. Clarification on the use of contrast agents would be valuable for reproducibility.
Furthermore, could the authors elaborate on the parameters used to determine abnormal areas of the kidney parenchyma? How were abnormal areas of the kidney parenchyma determined? I see that a deep learning model was used, but I do not have a clear understanding of how abnormal areas of the kidney parenchyma were segmented and distinguished from normal renal parenchyma. The appearance of the renal parenchyma also depends on the phase of the scan (arterial, nephrographic, or secretory). Additionally, which phase of the scan (arterial, nephrographic, or secretory) was used for segmentation in this study? Understanding these details is crucial as the phase of the contrast would not affect the kidney size but could influence the appearance of the renal parenchyma.

In conclusion, I believe addressing these points would further enhance the understanding and reproducibility of the study. Thank you for considering my comments.

Sincerely,

Diana Kaya
Assistant Professor
Department of Neuroradiology
UT MD Anderson Cancer Center

Point-by-point Response to the Reviewer's Comments on the manuscript "Multiscale and Multimodal Evaluation of Autosomal Dominant Polycystic Kidney Disease Development"

The reviewer's comments are shown in black, and the answers from the authors in blue.

Reviewer #1 (Remarks to the Author):

This study performs a detailed analysis of Pkd2 cko mice induced for Pkd2 loss starting at 4 wks. The animals are analyzed histologically, for survival, and by MR and CT imaging. The study provides quite good analysis of the progression in this model.

Specific points:
1. The animal numbers per group are very small 3 per sex for many endpoints, making it difficult to be precise about the course of disease development. This especially true because of variably between animals.

We acknowledge the reviewer's concern regarding the small number of animals per group. The development of the pathological model posed significant challenges. Breeding a sufficient number of mice with the required genetic modifications was a complex process, and we were only able to produce a limited cohort. Additionally, the logistics of our study involved transporting the animals to a different city for CT and MRI scans. This transportation and relocation process unfortunately contributed to increased mortality among the mice, further reducing our sample size for these specific analyses.

We recognize that a larger sample size would provide more robust statistical power and reduce the impact of inter-animal variability, which has been included as a limitation in the **Discussion** section, in the new version of the manuscript (lines 310 to 313):

"*Breeding a sufficient number of mice from the animal model was a complex process, which together with the need to relocate some of the specimens to a different city for CT and MRI imaging, caused some alterations on their development, unfortunately contributing to increased mortality. This in turn reduced our sample sizes for these analyses.*"

Despite these limitations, we believe our study provides valuable insights into the progression of ADPKD and sets the groundwork for further research with larger cohorts. Future studies will aim to mitigate these issues by improving breeding protocols, optimizing transportation procedures to minimize stress and mortality, and seeking additional resources to increase the number of animals in each group.

2. Automated methods for determining TKV have been described by only semiautomated methods are described here.

We appreciate the reviewer's comment regarding the use of automated methods. In our study, the primary objective was to achieve highly accurate segmentations of the entire organ. Although we initially tested fully automated methods, we encountered challenges, particularly with kidneys in the latest stages of disease. These kidneys exhibited significantly large cystic areas that were often connected to the boundaries of the organ, complicating the extraction of accurate masks.

To address these challenges and ensure the reliability of our results, we opted for a semiautomated approach. This method allowed us to manually review and correct the masks generated by the automated process, thereby mitigating the risk of mis-segmentation. We believe that this approach provided more precise and dependable segmentations, which are crucial for the accurate assessment of disease progression.

3. The accuracy of the CT masks to determine cystic regions is uncertain as these volumes seem much more variable than the TKV measurements.

For our study, while histology images provided a high-resolution view of the kidneys, enabling detailed visualization of cysts, CT scans allowed for volumetric monitoring over time, albeit with less detail. This inherent lower resolution and the challenges in segmenting small or poorly contrasted cysts likely contributed to the observed variability in CT-based measurements. However, the primary aim of CT scans was to estimate the overall disease progression within the entire kidney volume and provide a reconstruction of the disease spreading through the organ.

Despite their variability, these measurements were cross-referenced with histological data to ensure consistency in observed disease trends. CT resolution was sufficient enough to provide the pertaining global measurements.

It is true, however, as noted by the reviewer, that a more detailed analysis of ADPKD development would benefit from more detailed volumetric reconstructions. Future studies will consider the need for higher accuracy 3D imaging that allows for more precise segmentations, as mentioned at the **Future Work** subsection within our revised manuscript (lines 338 to 340):

"For future endeavors, the integration of advanced high-resolution 3D imaging techniques, such as Light Sheet Fluorescence Microscopy, could enhance the precision of volumetric analyses of cysts, enabling deeper exploration of their shapes and distribution patterns."

4. It is not clear why animals with more severe disease, 12 wks and later after induction, were not imaged.

As mentioned previously, the development of the pathological model encountered certain difficulties. The relocation of animals and variations in breeding conditions for those assigned to CT and MRI acquisition, compared to those used for histology, likely influenced their behavior as the disease progressed. Thus, higher mortality rates at earlier stages of the disease reduced the number of available specimens, making it challenging to acquire reliable imaging data at 12 weeks and beyond. Consequently, the limited number of surviving animals prevented us from obtaining sufficient samples for consistent imaging at these later stages.

5. It is unclear why when using a 7-Tesla Biospec 70/20 scanner that the resolution is too low to measure TKV?

The histology images obtained during this project provided a detailed view of specific cysts within the kidney, while the CT scans allowed a reconstruction of the whole organ. Our objective for MRI was to provide numbers of individual cysts within the whole kidney to complement the other modalities. The MRI acquisition was commissioned as a service with a limited available time, and thus we restricted the acquisition to a sufficient dataset for the assigned task. We acknowledge the reviewer's comment that a more detailed MRI acquisition could facilitate additional analyses on kidney structure, including TKV measurement. This consideration is mentioned in the **Future Work** subsection of our manuscript (lines 340 to 342):

"*Additionally, the use of higher-resolution MRI could provide a more granular temporal and volumetric view of the kidneys in specific mouse models, significantly improving our capacity to monitor disease progression with increased detail and accuracy.*

6. The model develops very quickly between 8 wks and 12 wks after induction. Were their efforts to analysis this period in more detail?

In our study, we observed the kidneys at specific intervals, separated by a fixed number of weeks. The measurements indicated rapid disease progression between 8 and 12 weeks. However, obtaining additional images at shorter intervals during this period was not feasible due to the logistical constraints of preparing additional mouse cohorts for more frequent studies within the scope of this project.

Future studies should indeed focus on a more detailed analysis of this critical period to capture the detailed progression of ADPKD. This would require dedicated cohorts and more frequent imaging sessions to provide a finer temporal resolution of disease development. This approach has been noted in the **Future Work** subsection of our revised manuscript as a crucial area for further research (lines 342 to 344):

"*For new studies, the focus could be shifted to a higher temporal resolution assessment of the stage starting from 8 weeks after induction. This could provide valuable information about the detailed evolution along this period, as it has been observed that most of ADPKD evolution occurs there.*"

7. Was Pkd2 expression monitored within the flox flanked deleted region? It would be more helpful to analyze PC2 protein level in the cko kidneys.

Yes, in our study we monitored Pkd2 expression within the flox flanked deleted region. We used specific PCR primers to quantify the wild-type mRNA expression, demonstrating a reduction of this RNA when Cre recombinase promoted the removal of exons 11-13, generating a null allele. We chose PCR analysis because it is more quantitative than Western blotting. However, specific quantification of PC2 protein levels in the cko kidneys was not performed. Nevertheless, analyzing PC2 protein levels could indeed offer additional insights into the impact of Pkd2 deletion on kidney pathology, as rightfully suggested by the reviewer.

Reviewer #2 (Remarks to the Author):

This is a very interesting manuscript that presents a workflow for assessing the progression of cystic regions in animal models of PKD using histology, CT and MRI.

1. The authors use the term "coalescent" and its meaning is unclear. Does this term mean that the cysts appear to "fuse" into a region because the distance between the cysts is below the resolution limit of the technique?

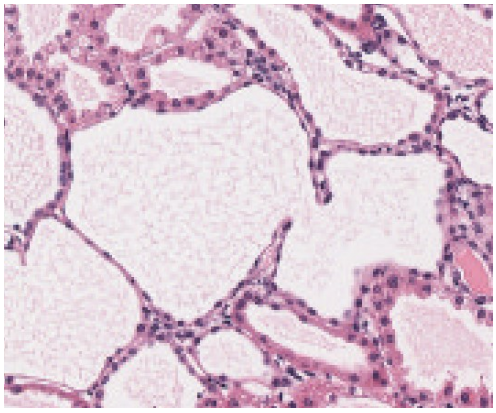
We appreciate the reviewer's insightful comment about the term "coalescent". As the disease progresses, an increasing number of cysts develop throughout the organ, and their sizes enlarge. Consequently, the boundaries between adjacent cysts diminish, eventually leading to the appearance of a continuous cystic region in our CT scans. Alternative modalities may be able to distinguish cyst boundaries at these stages, but our volumetric data showed these as homogeneous regions. Yes, this effect is mostly due to the proximity of the cysts falling below the resolution limit of the imaging technique.

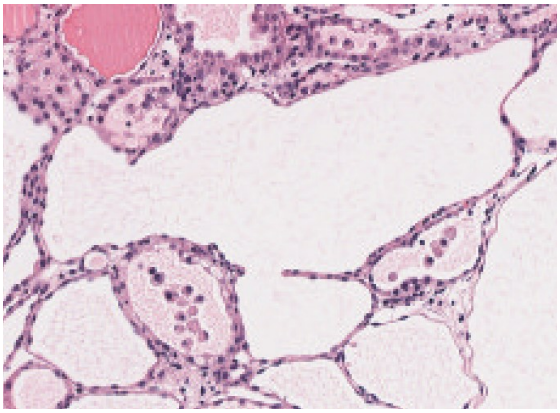
However, histology images support this observation by showing how the boundaries between cysts can be disrupted at times, leading to the fusion of smaller cysts into larger structures, which contributes to this phenomenon. We have clarified this explanation in the revised version of the manuscript and replaced the term "coalesce" by “merge”, to ensure it is accurately understood in the context of our study. The added information can be seen in the **CT and MRI Imaging Techniques for ADPKD Progression Analysis** subsection (lines 140 to 144):

“*In the course of ADPKD, our CT images reveal the progression of small cysts merging into larger affected regions, disrupting normal kidney functions. This is caused by groups of nearby cysts that grow adjacent to each other, creating areas in the kidney that are formed by only cysts, separated by thin walls. These are detected as homogeneous areas in our CT analysis, which is also supported by observations in histology images of cystic walls breaking so that several nearby cysts become combined to form a larger one.*”

2. Is cyst fusion something that is described or seen in histology of PKD?

Yes, as mentioned in the previous answer, cyst fusion is observed in histology images at some points of ADPKD development. The thin walls between cysts break and several of them are combined to form larger regions. This can be seen in cases were several cysts seem to merge into larger ones as the walls surrounding them are disrupted. The following images have been added to the supplementary material as examples of this phenomenon.





3. Can these methods used distinguish between normal interstitium and fibrosis?

Thank you for your interesting question. During the progression of ADPKD, the formation of cysts is indeed accompanied by the development of fibrotic tissue. However, the primary focus of our project was on analyzing the evolution of cysts, their size, and their impact on the overall kidney structure. In our CT and MRI images, it is not possible to distinguish fibrotic areas in detail.

Nevertheless, our histology images do reveal that cysts are often surrounded by fibrotic areas, and these regions develop progressively over time. While our current study did not specifically focus on segmenting fibrotic regions, this is an area of significant interest for future research. We recognize the importance of distinguishing between normal interstitium and fibrosis and plan to explore suitable segmentation methods for this purpose in subsequent ADPKD studies.

We have included a mention of this topic in the **Future Work** subsection of our revised manuscript to highlight its relevance and potential for future investigation (lines 344 to 347):

"*Another interesting direction to be explored is the analysis of fibrotic areas, besides cysts. A similar multimodal evaluation to the one performed during this project could help understand the behavior of the surrounding interstitium, although it would require higher resolution volumetric imaging.*"

4. Can these methods distinguish or detect calcifications such as kidney stone disease or Randall's plaques?

The reviewer's question is very pertinent and raises an important aspect. Unfortunately, the segmentation of calcifications falls outside the scope of our current project, which is specifically focused on the analysis of polycystic kidney disease. Our methods are designed to segment and quantify cysts, and we cannot definitively comment on their precision if applied to the segmentation of calcifications or other analyses.

However, similar methods to those we have applied could potentially be adapted for the detection and segmentation of calcification. This represents an interesting direction for future research, which could yield valuable insights into calcification-related kidney diseases. We appreciate the reviewer's comment and will consider exploring this application in subsequent studies.

Reviewer #3 (Remarks to the Author):

Dear Editor,

I am writing in response to the manuscript titled " Multiscale and Multimodal Evaluation of Autosomal Dominant Polycystic Kidney Disease Development" by Pablo Delgado-Rodriguez, Nicolás Lamanna-Rama, Cassondra Saande , Rafael Aldabe , María Luisa Soto-Montenegro , Arrate Munoz-Barrutia.

I would like to commend the authors for their insightful study. The study workflow represents a comprehensive approach for assessing the progression of PKD using CT, MRI, and histology, and the simultaneous utilization of these modalities is quite unique. However, I have some concerns and additional points that I believe could contribute to the discussion.

1. Firstly, I noticed that the use of a contrast agent in the CT evaluation of the kidney parenchyma was not mentioned in the Materials and Methods section. In Figure 6, a contrast agent appears to be present in the collective system of the kidneys. Clarification on the use of contrast agents would be valuable for reproducibility.

Thank you for bringing this to our attention. Indeed, a contrast agent was used during the CT acquisition to enhance the visualization of the kidney parenchyma. Specifically, Iopamiro (300 mg/ml, Iopamidol, Bracco Imaging S.p.A, Italy) was administered intravenously to the animals via the tail vein at a dosage of 0.25 ml. Imaging was performed 5 minutes after the administration to allow for adequate distribution of the contrast agent in the region of interest.

We have now updated the **Samples for CT and MRI Imaging** subsection of our Methods section to include this important information, ensuring that all details regarding image acquisition are thoroughly documented for reproducibility (lines 96 to 100):

"*For the CT study, Iopamiro (300 mg/ml, Iopamidol, Bracco Imaging S.p.A, Italy) was administered intravenously via tail vein (0.25 ml). After 5 minutes of its distribution, CT images were acquired under sevoflurane-inhaled anesthesia using a small-animal ARGUS PET/CT*

scanner (SEDECAL, Spain), with the following parameters: 340 mA, 40 kV, 360 projections, 8 shots and 200 μ m of resolution."

2. Furthermore, could the authors elaborate on the parameters used to determine abnormal areas of the kidney parenchyma? How were abnormal areas of the kidney parenchyma determined? I see that a deep learning model was used, but I do not have a clear understanding of how abnormal areas of the kidney parenchyma were segmented and distinguished from normal renal parenchyma.

To determine abnormal areas of the kidney parenchyma, we utilized a deep learning model, specifically a U-net convolutional network, to produce initial segmentations of the entire kidney. However, at later stages of the disease, significant degradation of kidney boundaries occurred due to the development of large cysts that extended to the edges of the organ, altering its contour. This made it challenging to extract precise masks using a fully automatic method. Therefore, we decided to manually correct the final segmentations to ensure more accurate measurements.

In the CT images obtained during this study, cysts formed by ADPKD appear as low-intensity areas, which increase in number and size over time. After obtaining the overall kidney volumes, the lowest intensity areas within these volumes were identified as damaged areas. This was achieved by first applying Otsu's method to automatically threshold the voxels within the kidney masks, removing very bright areas marked by the contrast agent, primarily within the renal pelvis. Subsequently, a second application of Otsu's thresholding was used over the remaining voxels to isolate the darkest regions, corresponding to the cystic areas. These segmented volumes were then manually reviewed and corrected for errors, ensuring the accuracy of the final measurements used to calculate the tissue volumes affected by the disease.

Thanks to the reviewer's comment, we have modified our explanation of this segmentation process in the revised manuscript, aiming to clarify the process as much as possible. This explanation appears at the **CT and MRI Imaging Techniques for ADPKD Progression Analysis** subsection of the new version of our document (lines 162 to 169):

"*Within the voxels marked by these full kidney masks, two Otsu thresholding [20] steps were implemented to delineate the damaged regions and track their progression over time. Otsu's method finds the optimal threshold value that maximizes inter-class variance between both classes of voxels, separated by being smaller than or larger or equal than that specific threshold. First, Otsu's method was applied to differentiate the high-intensity areas, marked by the contrast agent mainly in the renal pelvis. These high intensity areas were discarded. On the remaining voxels, a second Otsu was applied to isolate the darkest parts of the image, which corresponded to the cystic areas, damaged by the disease. This process facilitates the quantification of the evolving affected volume. The accuracy of the segmented diseased regions was further verified and adjusted manually to ensure precise measurement of disease progression.*

…

[20] N. Otsu et al., "A threshold selection method from gray-level histograms," Automatica, vol. 11, no. 285-296, pp. 23–27, 1975.

"

3. The appearance of the renal parenchyma also depends on the phase of the scan (arterial, nephrographic, or secretory). Additionally, which phase of the scan (arterial, nephrographic, or secretory) was used for segmentation in this study? Understanding these details is crucial as the phase of the contrast would not affect the kidney size but could influence the appearance of the renal parenchyma.

Thank you for this insightful question. The contrast agent used in our study, Iopamiro, is rapidly processed by the mouse body. At the time of imaging, which was performed 5 minutes after injection, the contrast agent had reached the nephrographic phase. This timing allowed for optimal visualization of the renal parenchyma.

Since our study focuses on the kidney, acquiring images during the nephrographic phase was crucial to ensure that the contrast agent effectively highlighted the renal structures. This clarification has been included in the **Samples for CT and MRI Imaging** subsection of the revised manuscript (lines 99 to 100):

"*Image acquisition was performed at the nephrographic phase of the contrast agent, aiming to enhance the visualization of kidney structures.*"

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

No new data has been added to the manuscript to address this reviewers concerns. However, some comments have been added to the manuscript acknowledging the data deficiencies.

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns.

Reviewer #3 (Remarks to the Author):

I have thoroughly reviewed the manuscript titled " Multiscale and Multimodal Evaluation of Autosomal Dominant Polycystic Kidney Disease Development" submitted by Pablo Delgado-Rodriguez. After careful consideration and major revision, I am pleased to recommend its acceptance for publication in Communication Biology. Here are my detailed comments:
The article presents original research that significantly contributes to the field of ADPKD in mouse models. The methodology includes simultaneous utilizing of histology, CT, MRI and application of automatic methods for full kidney segmentation.
The manuscript is well-written, with a clear and coherent structure.
The authors have clearly described their research design, data collection, and analysis procedures.
The results are presented in a logical manner, supported by adequate data and statistical analysis.
The references are current and relevant, demonstrating a thorough review of the pertinent literature.
Sincerely,

Diana Kaya