Dear editors and reviewer,

We hereby submit the revised version of manuscript WJG33732.

Thank you for considering our manuscript for your journal. We have read your comments carefully and addressed each point separately.

In addition, we included a copy of the revised manuscript with marked changes in yellow for your convenience.

Reviewer Name: Anonymous

Reivew Time: 2017-03-16 08:49

Comments to Authors:

In this study, authors have shown that everolimus halts hepatic cystogenesis in a rodent model of polycystic liver disease. They have developed a MRI-based method for accurate determination of liver volume and investigated that everolimus halted cyst growth comparable to lanreotide and reduced the development of fibrosis, mTOR-inhibition should be further explored in PCLD patients especially those that need immunosuppression. The study has been well performed but I have major comments as mentioned below:

1. Liver fibrosis was detected by picrosirius red staining in this study. But loss of stress fibers should be shown using phalloidin/actin stress fiber and α-SMA staining.

Reply: *- We appreciate the suggestion made by the reviewer. The proposed techniques can be used to investigate certain aspect in the cells more deeply and has shown its potential in many studies. When looking carefully at the literature and the information provided by the leading companies that provide reagents for this technique; we concluded that it is well suited for use on cells in culture. However, as described in our manuscript we conducted a longitudinal translational study on rats that showed progressive liver disease with formation of fibrosis and immune cell infiltration. We collected the tissue at the end of the experiments for histological, protein and gene expression studies. The way the tissue was fixed and processed is unsuitable for the here proposed technique. In addition, due to the heterogeneity of the cells in the section (in contrast to monolayer cultures from cells in vitro), we have no knowledge of a standard protocol that allows the use phalloidin/actin fluorescent staining on tissue section.*

2. The gene expression of SSTR2, SSTR5, mTOR and Rptor was analyzed by quantitative RT-PCR in this study. I think that protein expression of these genes should be shown using western blot.

Reply*: - To answer this comment we should divide our reply into two separate parts, as we will explain below. We agree that next to PCR also*

protein expression (and phosphorylation) are important aspects to understand the functional response of the animals under treatment. A) Therefore, with regard to the mTOR signaling pathway (schematic represented in figure 4B) we have investigated several key factors. Using PCR we investigated expression of mTOR and Rptor (figure 5B). In addition, and in line with the comment made by the reviewer, we also investigated by Western Blot technique two additional elements of the mTOR signaling pathway. We studied the phosphorylation of Akt, that is located before mTOR and we also looked at the phosphorylation of S6, a downstream element that is crucial for passing the signal within the cell. We think by investigating multiple connected elements we have given a logic presentation of what happens in these animals under lanreotide or everolimus treatment. We agree that additional in-depth molecular investigations are possible but those fall outside the scope of this more translational manuscript with emphasis on the imaging aspect. B) A major problem regarding the suggestion to study SSTR2 and SSTR5 is of a technical nature: in contrast to PCR-primers that can be designed with very high specificity and sensitivity, antibodies are offend limited in availability, their species specificity and sensitivity. Regarding the available antibodies for SSTR's (anti-SSTR2: HPA007264; Sigma and anti-SSTR5: PA3-112; Thermo), in our hands they detect multiple bands which is most likely because they are of polyclonal origin and require high protein loading and low dilution. Furthermore, as can be concluded from figure 5, the Ct values for SSTR2 and SSTR5 are high, which points to very low expression at the end of the treatment period for all conditions. Taking these limitations together, at this moment, the technique that is best suited to us for detection of changes in expression of SSTR-subtypes is the PCR.

3. Whether authors can add the clinical data of everolimus to this article in PCLD patients.

Reply: *- In a review we published the current knowledge on management of PCLD in patients (Temmerman F, Missiaen L, Bammens B, Laleman W, Cassiman D, Verslype C, van Pelt J, Nevens F. Systematic review: the pathophysiology and management of polycystic liver disease. Aliment Pharmacol Ther. 2011 Oct;34(7):702-13.). PCLD, being a relative rare disease, randomized trials using everolimus are lacking at this moment and data on the clinical use of everolimus in PCLD patients is very limited and conflicting. In the most recent review on this subject by Kahn et al. (Khan S, Dennison A, Garcea G. Medical therapy for polycystic liver disease. Ann R Coll Surg Engl. 2016 Jan;98(1):18-23.), they only found one study (reference 20), in which the added effect of everolimus in combination with somatostatin analogues was investigated and where it did not show additional significant effects. A statement on the available clinical data has been added to the discussion. We think it will be an important step if the findings of Crispijn et al. and of monotherapy with everolimus can be evaluated in a large-enough randomized trial. The relative small group of patients that offer themselves at the different*

hospitals with PCLD, its slow progression and long period of treatment required make it difficult to perform such a study. Therefore, preclinical studies using the here presented rat model and longitudinal follow-up can help to select the most promising approach before going to the patients.

Classification:	Grade C (Good)
Language Evaluation:	Grade B: minor language polishing
Conclusion:	Major revision
Confidential Comments To Editor(File):	
Comments To Authors(File):	

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Comments raised by the journal:
Please provide language certificate letter by professional English language editing companies (Classification of manuscript language quality evaluation is B).

- *Dear Sirs,*

We have great respect for the work of the reviewers and editors. However, on this point we disagree. We need to mention that we all have had our higher education in English, in addition we have worked for a long time in an international context using English on a daily basis and some of us even worked for a period as post-doc in London at the Imperial College. Therefore we find this suggestion misplaced. Over a period of 30 years, each of the PIs have written more than 100 peer reviewed articles that have been published in high-impact journals and frequently have given talks at international conferences in the US, Europe, Asia and Africa.

We can imagine that certain sentences can be formulated always in more than one way, but polishing the English in this manuscript will be more related to individual perception of how things could be written (either US-UK-Australia-etc.). Following your remark, we have considered the entire text and have made some changes to the manuscript and have the text reviewed by two other senior PIs. We hope this is to the satisfaction of the editors.

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Institutional review board statement:

NA

Informed consent statement:

NA

Institutional animal care and use committee statement:

KU Leuven, P164/2010

Animal care and use statement:

10-week-old female PCK rats were purchased from Charles River, France). The animals were housed in the central animal facility of KU Leuven and cared for by dedicated animal technicians according the guidelines approved by the university committee for animal welfare.

Biostatistics statement:

Data were analyzed using MedCalc version 14.12.0 (Medcalc, Ostend, Belgium: http://www.medcalc.org).

Conflict-of-interest statement:

None to declare

Data sharing statement:

NA

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Core tip:

The continuous volume increase of liver cysts in polycystic-liver-disease (PCLD) leads to extensive hepatomegaly and portal hypertension, an indication for liver transplantation. The effect of mTOR-inhibition on liver volume (LV) in PCLD is unclear. We developed an accurate, non-invasive, MRI-based method to determine LV in a PCLD rat model. When treatment is started at the moment of extensive hepatomegaly (as in humans), the mTOR inhibitor everolimus halts disease progression and also the development of fibrosis in this model. We speculate that everolimus, given after kidney transplantation in patients with PCLD, can prevent the development of symptomatic hepatomegaly.

Audio core tip:

Attached as a separate file

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Remark: Please provide the decomposable figure of Figures, whose parts are movable and editable. So please put the original picture as PPT so that we can edit them easily.

Reply

- Dear sirs, we have added a high-resolution image (300 dpi, TIFF-format) of the 5 figures of this manuscript. In addition, we have provided also a PPT-file of the original figures as requested.

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1 What did this study explore?

In this study, the authors developed and validated a non-invasive MRI-based method for the assessment of liver volume in rats. This method was subsequently used to study the effect of mTOR-inhibition on liver enlargement in the PCK rat model for polycystic-liver-disease in a clinically relevant study design.

2 How did the authors perform all experiments?

There are three critical steps in this study: a) to adopt the well-established, genetic rat model for human PCLD; b) to establish and statistically validate an MRI-based method for longitudinal follow-up of LV in the model; and c) to use a clinically relevant study design, where the treatment starts at the moment of proven hepatomegaly and a somatostatin-analogue (lanreotide) used in patients is applied as control.

3 How did the authors process all experimental data?

Liver volume was determined using non-invasive methods. At the end of the treatment the method was statistically correlated with ex-vivo volume measurements. Gene and protein expression was determined of complementary members of the mTOR signaling cascade, whereby the level of phosphorylation by Western blot (relevant for the biological signaling) was given extra attention.

4 How did the authors deal with the pre-study hypothesis?

Based on literature and KEGG-pathways, relevant molecular markers were selected.

We compared the findings to what could be found using untreated (negative control) and lanreotide (positive/clinical relevant control).

Using a study design that takes into account the clinical aspects (start of treatment once hepatomegaly has developed and individual follow-up to take into account inter-individual variations) we could address the pre-study hypothesis.

5 What are the novel findings of this study?

- *We developed a unique MRI-based method that was shown to be sensitive and statistically reliable.*
- *Repeat measurements allowed for monitoring individual responses and reducing the number of animals required for this type of study.*

- *This method is a great step forward for it allows preclinical testing of drugs for PCLD under controlled conditions that cannot be achieved in humans mainly for logistic and time considerations.*
- *Everolimus looks beneficial to reduce the cyst volume in PCLD with a secondary benefit on fibrosis.*
- *Polycistic liver and kidney diseases are closely related. Kidney transplantation is a frequent treatment for ADPKD (Autosomal dominant polycystic kidney disease (ADPKD, autosomal dominant PKD or adult-onset PKD), and this study gives arguments to use everolimus in patients that need immunosuppression as it can have beneficial effects for the livers at risk in ADPKD patients.*

At last, sign (handwrite) your name in the end of file.