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RESEARCH ARTICLE

Monitoring kidney and renal cyst volumes applying MR approaches on a rapamycin treated mouse model of ADPKD

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Abstract
Object The aim of our study was to determine total cystic volume in a mouse model of PKD using MR imaging to monitor therapeutic effects in vivo.
Materials and methods We imaged eight female *pcy*-mice in two groups: four belonged to an untreated control group and four were treated with the anticystic agent rapamycin, which has proven to be effective in reducing cystogenesis in animal models. The mice were imaged using a 9.4 Tesla animal scanner. MRI measurements were taken at six time points during the therapy. Total renal volumes and total cyst volumes were calculated using a thresholding approach.
Results During the course of the treatment, the total cyst volume increased significantly faster than the total renal volume in the untreated group, indicating that growth of the total renal volume in the untreated group was primarily due to the growth of the cysts, rather than the parenchyma. The measured total renal volume in the control (placebo) group was significantly higher than the volume in the treated group.
Conclusion Using MRI, we were able to monitor the cystic volume in a mouse model of PKD to assess the therapeutic effect of anticystic treatment.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common monogenetic disorders, affecting about 12 million people worldwide. Excessive proliferation of renal epithelial cells and the transformation from an absorbing to a secreting epithelium leads to cyst propagation and enlargement that eventually replaces most of the normal surrounding tissue and is accompanied by higher apoptosis rates [1,2].

ADPKD results in severe enlargement of the kidneys, with end stage renal failure occurring in most cases by the age of 50, consequently leading to kidney transplantation or lifelong haemodialysis. In addition to the deterioration of renal function, renal cysts may cause pain and other symptoms resulting from mechanical compression of adjacent organs. ADPKD is also associated with hypertension, hematuria, cyst infection and renal stones [3]. Recent developments showed the possibility of pharmaceutical treatment of ADPKD to decelerate the growth of the cyst, protracting the onset of haemodialysis [4,5]. There are several animal models for PKD in mice [6,7] and rats [8]. The established *pcy*-model [7] was first described as a spontaneous mutation of the KK strain background, a known diabetic strain. The clinical course and morphology of the slowly growing murine kidneys resemble the human adult form of polycystic kidney disease. Fluid filled renal cysts can be identified in all segments of the nephron and collecting duct and are progressively enlarged with age. Individual cysts can be found to be lined by a single layer of epithelial cells in most areas [9,10]. Recently Olbrich et al. proposed that a homozygous missense mutation in the Nphp3 locus on 3q21–q22 is responsible for the polycystic kidney disease (*pcy*) mouse phenotype [11].

MRI has already proven to be a reliable method to measure renal volumetric indices in preclinical [4,12,13] and clinical studies [14–17]. Therefore, MRI-based volume measurements can be used as a reliable medium to detect the progression of the disease. Previously, only renal volume has been used in animal models to monitor treatment effects. To study the development of the cyst volume (CV) in comparison to the total renal volume (RV), we have initiated this in vivo MRI study using Rapamycin as a therapeutic agent, which has proven potential to ameliorate cyst progression in rodent models [4].

Materials and methods

Eight female *pcy*-mice (12 weeks old) were distributed into two groups of four mice. One group was treated with a 5 mg/kg per day rapamycin dissolved in 21.4% dimethylsulfoxid (DMSO), 21.4% ethanol, and 57.2% isotonic sodium chloride solution. The second group was vehicle treated. Rapamycin was chosen as a therapeutic compound for this study, because of its well described effects on cystogenesis in rodent models [4,5]. Rapamycin was applied at regular 24-h intervals intraperitoneally with a concentration of 5 mg/kg per day, as reported previously [4]. All animals were surveyed on the same day every 2 weeks during the 10-week treatment.

Mouse model

Mice bearing the *pcy/pcy* genotype were maintained in a temperature-controlled room, with a light/dark cycle of 12 h/12 h (lights on at 0700 hours), and were randomly assigned to experimental groups before the treatment. All experiments were performed in accordance with the local animal care commission.

Histochemical analysis

A histochemical analysis was performed to correlate MR findings of the renal cystic volumes with histology. The explanted mouse kidneys were immediately equilibrated at 4°C over night in 0.6 M sucrose/phosphate-buffered-saline (PBS). Prior to sectioning, the organs were dehydrated in an ascending alcohol sequence, then transferred into xylol and finally embedded in paraffin blocks. Sections of size 5 µm were cut from each kidney. After rehydration, each section was stained with hematoxylin for 5 min and after extensive washing with tap water the sections were morphologically examined with a Zeiss microscope (Zeiss, Oberkochen, Germany).

Histological relative cystic index calculations

After the animals had been sacrificed, one animal per group was chosen for histology. For each animal, three representative histologic images of hematoxylin and eosin-stained kidneys were acquired. Regions of interest (ROI) were drawn using the freely available MIPAV software tool (National Institutes of Health, Bethesda, USA). To quantify the relative area of the cysts in the histologic sections, the cysts were delineated from renal parenchyma on representative histological sections, and cystic area was calculated by summing up the individual areas of the ROIs. The percentage of cystic area was then determined from the ratio of total cystic area versus total renal area on the slice.

MR imaging

Due to logistic challenges measurements took place on 14-day intervals from day 35 of the treatment (day 123 postpartum) until the end of the treatment (day 193 postpartum). We performed in vivo measurements in the control and rapamycin-treated groups to determine the total kidney volume and the total volume of cysts at designated time points during therapy. A total of eight mice, four in each experimental group, were imaged with a 9.4 Tesla small bore animal scanner (BioSpec 94/20, Bruker Biospin, Ettlingen, Germany) using a cylindrical quadrature birdcage resonator with an inner diameter of 38 mm, specifically designed for whole body mouse imaging.

Mice were anesthetized under spontaneous breathing conditions using isoflurane. Heart rate and respiration rate were continuously monitored and kept at a constant level. Gating was used to reduce motion and blood flow artefacts during the scan. MR measurements were taken at six distinct time points postpartum (Figs. 3, 4: d123, d137, d151, d165, d179 and d193) within the therapy.

The MRI protocol consisted of a T1-weighted FLASH (Fast Low Angle Shot) gradient echo sequence as a fast imaging technique to outline the renal borders (TR/TEeff/FA: 350 ms/5.4 ms/40°). A fluid-sensitive T2-weighted spin echo RARE (Rapid Acquisition with Relaxation Enhancement) sequence was performed to delineate the renal cysts from the surrounding renal parenchyma (TR/TEeff/FA: 3,000 ms/ 36 ms/180°, echo train length: 8). Both sequences featured a FOV of 30 × 30 mm^2; a matrix size of 256 × 256 $pixel^2$, and an in-plane resolution of 117 × 117 μm^2. The slice thickness was 0.5 mm with no slice spacing to achieve contiguous image sets of the whole volume (Fig. 1). The number of slices was adjusted to the measured volume (on average 25) to ensure complete coverage of the kidneys.

Measurements of cyst volume and total renal volume

The kidney volume estimation technique involved a manual segmentation (perimeter drawing) of kidneys excluding the calices and the renal pelvis, which could be clearly

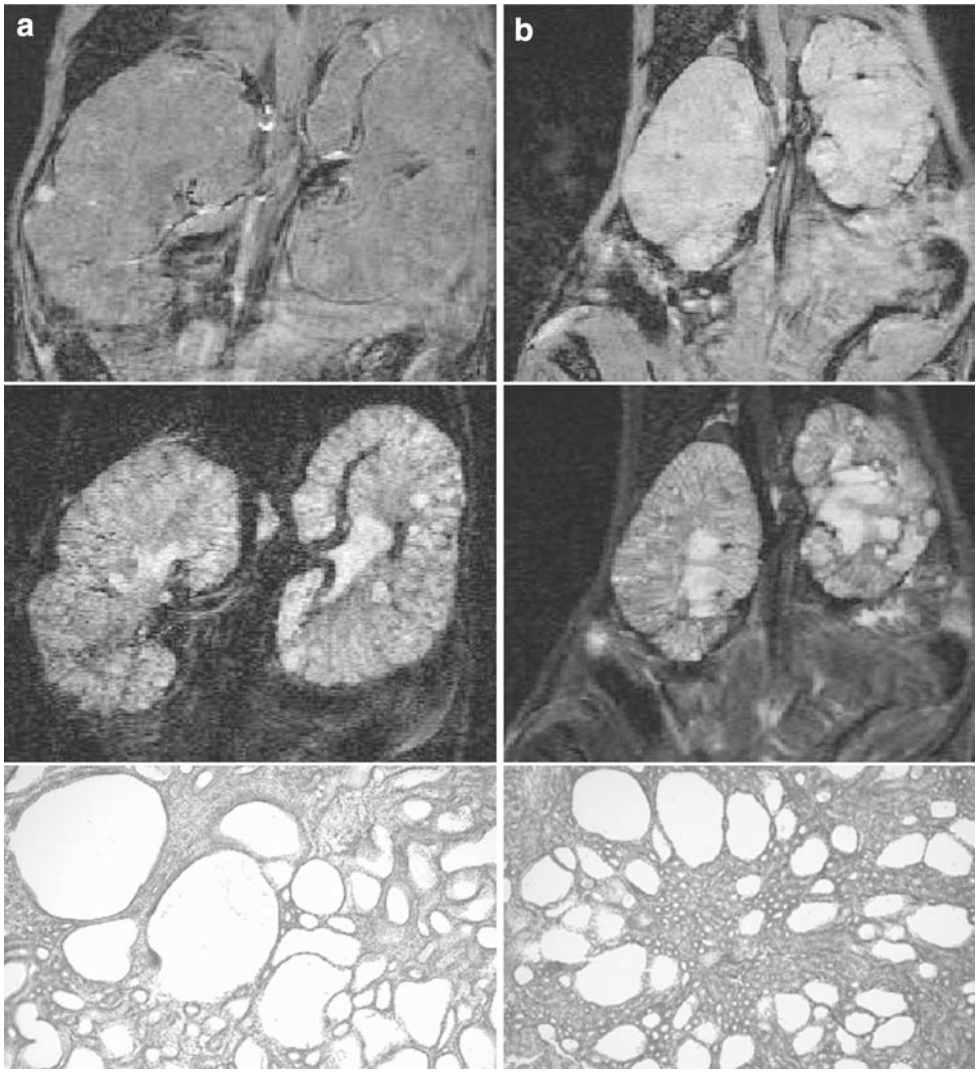

Fig. 1 *Upper row*: FLASH Images with TE = 5.4 ms of untreated *pcy*-mouse model (**a**) and rapamycin treated *pcy*-mouse model (**b**). *Middle row*: RARE Images with TEeff = 36 ms. *Lower row*: corresponding histological sections

distinguished from the surrounding parenchyma and the cysts (Fig. 1), and a semiautomatic threshold approach using an analysis of threshold signal intensities of renal parenchyma and cysts. For this method the perimeter of the kidney was manually traced on each slice image. The area of the cysts within the segmented kidney was obtained by a region-based threshold method. The total RV was calculated from sets of contiguous images by summing up the products of area measurements and slice thickness using a Matlab-based, custom-built software application. The percentage of CV was determined from the ratio of CV versus RV in each kidney separately.

Figure 2 shows the placement of the ROIs and a false color visualization of the signal intensity values for histogram analysis. The threshold was set individually for each examination based on the corresponding histograms and images by targeting threshold values designating the transition between parenchyma and cyst that could be detected at the border of larger cysts in the kidneys.

The reliability and reproducibility of histogram based techniques in quantifying renal cysts in human studies has been previously shown [14].

Statistics

The data are expressed as mean ± SEM. Statistical analysis was performed by two-factor repeated measure ANOVA using SigmaStat® 3.5 (Sysstat Software Inc., Germany). Significant overall effects were followed by pair-wise comparison using the Holm–Sidak t-procedure. The level of significance was defined at $P < 0.05$ (two-tailed). Error bars in Figs. 3, 4, and 5 represent the standard error of the mean. Calculations on body weight and kidney weight of the animals were done using an unpaired t-test.

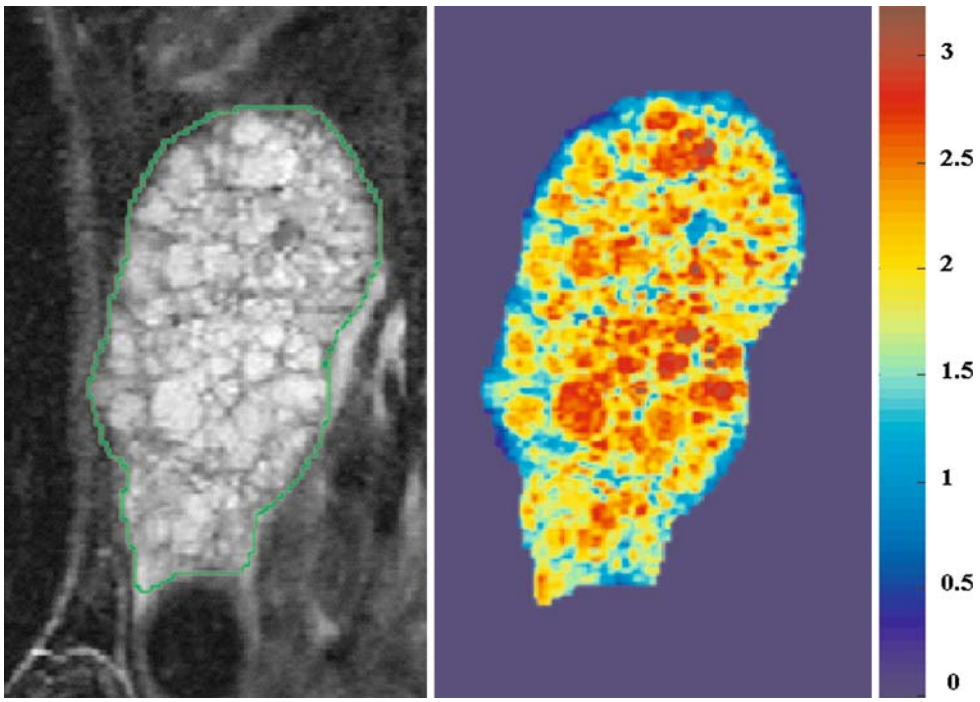

Fig. 2 Renal volume estimation technique involving manual segmentation of kidneys and a semiautomatic thresholding approach

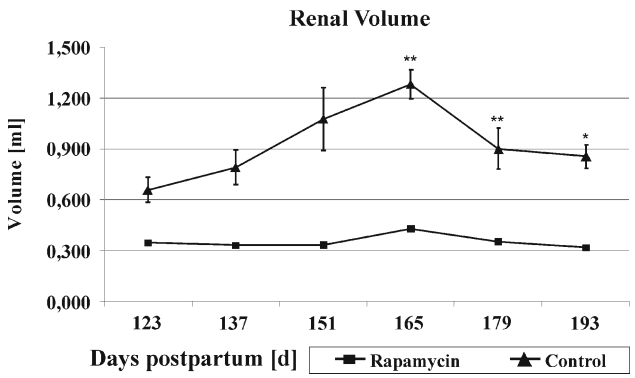

Fig. 3 Total Renal volume in ml during therapy. Values shown are averages, with error bars representing ± SD, ($P < 0.05 = {}^{*}$, $P < 0.01 = {}^{**}$)

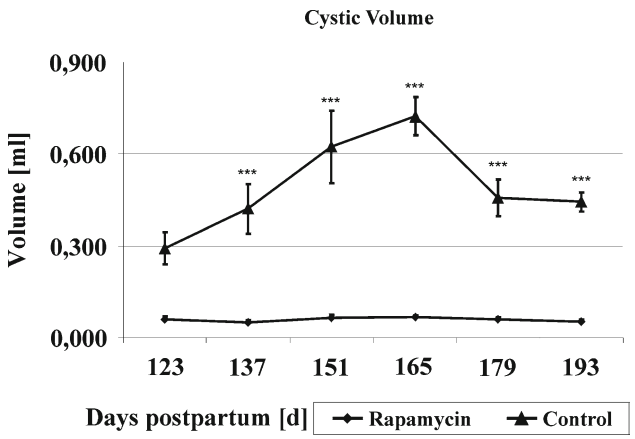

Fig. 4 Cystic volume in ml during therapy. Values shown are averages, with error bars representing ± SEM, ($P < 0.001 = {}^{***}$)

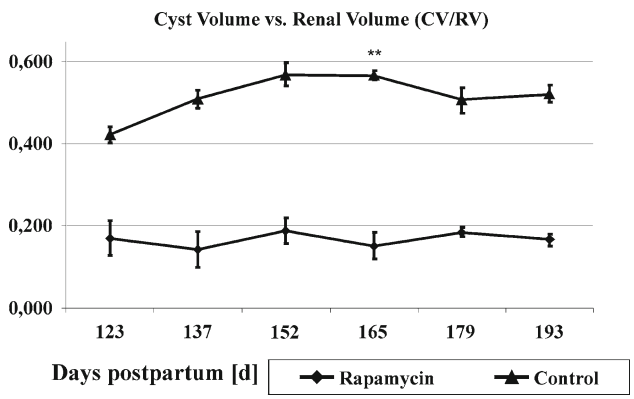

Fig. 5 Cystic volume versus total renal volume (CV/RV ratio). Values shown are averages, with error bars representing ± SEM, ($P < 0.01 = {}^{**}$)

Results

Volumetric measurements

The measured CV and RV in the rapamycin group showed no statistically significant differences during treatment and stayed at a stable level throughout the therapy.

A significant ($F = 5.078$, $P < 0.05$) difference in the measured RV (Fig. 3) between the control and treatment experimental groups was observed. The RV was initially higher in the control group on day 123 than in the treated group and increased significantly until day 165. In compar-

ison to the rapamycin group, the control group RV was significantly higher during day 137 to day 179 ($P < 0.01$). Following the initial increase we observed a decrease in RV until the mice were sacrificed on day 193 postpartum. The change of the CV in the untreated control group paralleled the change in RV (Fig. 4) and the difference between the groups was highly significant ($F = 28.039$, $P < 0.001$) from day 137 until the end of the treatment (day 193 postpartum).

Ratio of CV versus RV (CV/RV ratio)

Overall, there was a significant difference ($F = 5.202$, $P < 0.039$) in the CV/RV ratio between the treatment and experimental groups. During the course of the rapamycin treatment, the CV/RV ratio increased in the control group until day 165 postpartum, before finally decreasing until day 193 postpartum. During the same period of time the CV/RV ratio in the rapamycin group remained stable and significantly lower on day l65 than the CV/RV ratio in the control group (Fig. 5).

Histology

The histologically determined relative area of the renal cysts versus the total renal area in the rapamycin group on day 193 postpartum was 11%, whereas the detected relative cystic area in the control group was 43%.

Mouse body and kidney weight

At day 193 of the treatment period the average weight of the mice in the treated group (17.5 g) was significantly ($P < 0.05$) lower than the average weight of the control group (22.5 g), while the average kidney weight in the control group (0.852 g) was significantly ($P < 0.05$) higher than the average kidney weight in the treated group (0.277 g). The percentage of kidney weight/body weight was higher in the control group (3.76%) than in the treated group (1.58%).

Discussion

Total renal volume is currently used in clinical applications as an important parameter for assessment of patients with kidney disease. Serial in vivo kidney measurements have also been shown to be valuable tools to monitor response to treatment and therefore serve as an indicator for treatment evaluation and the basis for therapeutic decisions [15,16].

Recent studies have illustrated the potential of MR to elucidate the development of renal cysts in the course of ADPKD in clinical studies [14,15] and also in animal models of cystic diseases [4,12,13,18]. Recently, Wallace et al. showed that in vivo measurements of kidney volume using a dedicated animal scanner can accurately monitor the increase of renal volume in disease models [18]. However, previous animal studies have not focused on the increase of total cystic volume as a distinct parameter during the monitoring of treatment. By establishing a new technique to monitor the CV in addition to the RV we were able to use this information as a surrogate marker for treatment. The measurement of CV made it possible to distinguish between therapeutic effects on renal cyst development and effects on total renal volume. Despite the fact that RV was also correlated with therapeutic response to rapamycin in the pcy-model, we still detected a statistically significant increase of the CV until day 165 of postpartum (Fig. 4). The observed decrease of the CV and the CV/RV ratio in the untreated control group from day 165 postpartum could be due to the increasing level of inflammation and fibrosis in the pcy-model [18,19]. As the body weight of the animals in the group treated with rapamycin was significantly lower in the treated group, the used dose of rapamycin seemed to affect the growth of the animals. Kidney weight and the percentage of kidney weight relative to the body weight were well correlated to the measured RV on day 193 postpartum confirming the MR measurements. In our model the statistically highly-significant increase of the CV until day 165 postpartum in the control group indicated that the observed growth of the total renal volume was primarily due to the growth of the cysts, rather than the parenchyma. Histology revealed significant differences between treated and non-treated groups, featuring large cysts in the renal parenchyma of the control group and smaller cysts in the rapamycin treated group, similar to the histological findings of other studies using rapamycin as an anticystic therapy in a different animal model [4]. Since the clinical cyst measurements with the described technique are well characterized we did not perform histological correlation of the MR measurements at each time point, since we would have lost too many animals for the longitudinal in vivo measurements. In contrast to other studies [17], we were also able to establish a method to determine the CV without using contrast agents. This is favorable clinically [14], mainly due to the recent findings linking the administration of gadolinium-containing contrast agents to nephrogenic systemic fibrosis (NSF) [20,21], which limits the use of contrast agents in ADPKD-patients.

Rodent models have been used extensively to study the pathophysiology of renal cystic diseases, especially polycystic kidney disease [6–8]. In recent years a number of new therapeutic agents have been found [22–25] and they are currently under clinical testing. It is valuable to have non-invasive tools to monitor the development of the cysts during novel therapeutic strategies. Since MRI can non-invasively measure the CV in living mice longitudinally, it will be possible to determine the development of the CV in individual

mice. Furthermore, it will be possible to monitor drug effects more rapidly and with greater detail compared to studies that measure only the total RV [4,12]. As the approach, that was used to calculate the CV/RV, could be performed on basis of the total renal ROIs, this step did require relatively low extra workload in the postprocessing (approximately 5 min). No additional scan-Time was necessary. Since the CV and the CV/RV ratio can be used as additional and more sensitive surrogate markers of the drug effect, we regarded the extra workload to be justified.

The cystic volume and the total renal volume measured by MRI were larger than those measured by histology on day 193. This difference could be explained by loss of cyst fluid during kidney sectioning, and tissue shrinkage due to the hypertonic solutions and dehydration alcohols used during paraffin embedding [18]. These effects could lead to a shift between the relative cystic volumes measured by histology and the in vivo determined ratio. The starting point for the MR investigations was another drawback of this study, as it did not coincide with the initiation of the treatment. Consequently, the RV and CV values for the two groups were initially different at the first MR imaging measurement. However, the goal was to monitor changes related to the therapy and the age of the *pcy*-mice, and therefore this discrepancy was considered acceptable. Furthermore, with respect to histology it is obvious that the size of a considerable fraction of the cysts may be smaller than the physical resolution of the MR images. Also, the range of the cyst sizes is very broad. This could result in partial volume effects compromising the discrimination of cysts and renal parenchyma, and therefore the exact determination of the CV.

Future preclinical studies will focus on different MR techniques, such as diffusion, that would reflect the dependence of diffusivity of the total kidney on total cystic volume. This might find additional morphological markers for the characterization of the cyst development in animal models.

This study presents a novel use of MR techniques to distinguish between total renal enlargement and cystic enlargement during disease progression under therapy. By assessing the CV and CV/RV, we could directly monitor the therapeutic effects of rapamycin on cyst progression over a given period of time.We therefore think, MRI is a reliable non-invasive tool to screen larger cohorts of rodent disease models and is a valuable tool to aid in the evaluation of new therapeutic approaches.

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