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Sirolimus Reduces Polycystic Liver Volume in ADPKD Patients After Renal Transplantation

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Abstract

Excessive proliferation of the biliary epithelium is a prominent feature of polycystic liver disease (PLD), for which there is no medical treatment. Sirolimus, an immunosuppressive agent, exerts an antiproliferative effect by inhibiting mammalian-target-of-rapamycin (mTOR).

We retrospectively measured the volumes of polycystic liver and kidneys in 16 ADPKD patients who participated in a prospective, randomized trial in kidney transplantation comparing sirolimus-mycophenalate mofetil-prednisone to tacrolimus-mycophenalate mofetil-prednisone and had abdominal imaging studies before (within 11 months or at initiation) and after (at least 7 months) receiving immunosuppressants. Treatment with the sirolimus-containing regimen in 7 patients for an average of 19.4 months (range 7-40) was associated with a reduction in polycystic liver volume by 11.9 ± 0.03%, contrasted with a 14.1 ± 0.09% increase ($P < 0.01$) in 9 patients received non-sirolimus regimen for a comparable duration. A trend towards a greater reduction in native cystic kidney volumes was also noted in the sirolimus (14.8 ± 0.08% and 13.8 ± 0.08%) compared to the non-sirolimus group (10.9 ± 0.06% and 9.0 ± 0.06%, right and left kidneys, respectively). Consistent with these observations, hepatic cyst-lining epithelium, when compared to the biliary epithelium in controls, exhibited marked elevations in phospho-AKT, phospho-ERK, phospho-mTOR, and its downstream effector phospho-S6rp.

In summary, PLD cyst-lining epithelium exhibits aberrant activation of mTOR signaling, likely contributing to the cystic liver enlargement. A sirolimus-containing immunosuppressive regimen is associated with a significant reduction in polycystic liver volume after renal transplantation. These observations suggest that sirolimus deserves consideration in the treatment of severe PLD.

Introduction:

Autosomal dominant polycystic kidney disease (ADPKD) is a life-threatening monogenic disease with a prevalence of 1 in 400-1000 live births. Polycystic liver disease (PLD) is its most common extra-renal manifestation present in the majority of ADPKD patients by age 60**(1, 2).**

ADPKD is caused by mutations to *PKD1* (approximately 85% of the cases) or *PKD2* (the remaining 15%) genes, encoding polycystin-1 (PC1) and polycystin-2 (PC2), respectively. PC1 is a putative cell-surface, receptor-like protein with yet-to-be-identified ligand(s), and PC2 a channel protein with a high conductance to Ca^{2+} (reviewed in**(3)**). PC1 and PC2 are expressed in multiple cellular systems including renal and biliary epithelia. Interaction of PC1 and PC2 in renal epithelial cells inhibits cell-cycle progression**(4)**. *PKD* mutations induce a change in renal epithelial cell phenotype associated with an activation in cAMP/Ras/Raf/ERK signaling**(5-8)**. Downstream to this aberrant signaling, mammalian-target-of-rapamycin (mTOR) is found to be activated and may contribute to excessive tubular epithelial cell proliferation and renal cyst expansion**(9).**

Sirolimus (rapamycin) is a macrocyclic lactone derived from a strain of streptomyces hygroscopicus. It inhibits cell growth/proliferation and promotes apoptosis by inhibiting mTOR-mediated signaling**(10, 11)**. Sirolimus has been used in renal transplant patients as a part of alternative long-term immunosuppressive regimen with a comparable or superior allograft outcome to that of calcineurin-containing immunosuppression**(12, 13)**. In recent years, its usage has been extended, experimentally and clinically, to the treatment of immune-mediated glomerulonephritis **(14)**, an array of tumors**(15-17)**, refractory uveitis, and coating stents to prevent coronary artery re-stenosis**(18, 19).** Recently, sirolimus has been shown to reduce cystic renal enlargement in animal models of PKD and the native end-stage cystic kidneys in ADPKD patients with a functional allograft after renal transplantation**(9, 20, 21).**

Liver cysts in ADPKD originate from biliary microhamatomas or focal proliferations of biliary ductules and from peribiliary glands. Excessive proliferation of biliary epithelial cells, combined with neovascularization, altered cell-extracellular matrix (ECM) interaction/ECM remodeling and cAMP-mediated fluid secretion, is required for the development and expansion of PLD liver cysts**(22-25).**

PLD may become symptomatic with acute complications such as cyst hemorrhage, rupture and infection. Chronic symptoms are frequently associated with massively enlarged PLD, including abdominal distension and pain; dyspnea; gastroesophageal reflux and early satiety which may lead to malnutrition; mechanical lower back pain; obstructions of the inferior vena cava, hepatic and portal veins (leading to dialysis associated hypotension, hepatic venous outflow obstruction, and portal hypertension) biliary obstruction. Currently, apart from invasive interventions such as cyst aspiration with sclerosis, cyst fenestration, combined hepatic resection and cyst fenestration, liver transplantation and, rarely, selective hepatic artery embolization, no medical treatment is available**(26)**.

We have retrospectively examined the effect of sirolimus on PLD in patients who participated in a prospective, randomized trial after kidney transplantation comparing sirolimus-mycophenalate mofetil-prednisone to tacrolimus-mycophenalate mofetil-prednisone, and had computed tomography (CT) or magnetic resonance imaging (MRI) of the abdomen prior to and after renal transplantation. The sirolimus-containing regimen for an average duration of 19.4 months was associated with a reduction in total liver volume, while the liver volumes continue to increase in patients received non-sirolimus regimen. Consistent with this observation, mTOR and its downstream effector, S6 ribosomal protein (S6rp), were highly activated in PLD cyst-lining epithelia, but not in non-ADPKD liver sections.

Methods:

Clinical Data Collection

The study was approved by the Institutional Review Board. The records of ADPKD patients, diagnosed by clinical criteria, who participated in a prospective, randomized trial in kidney transplantation comparing sirolimus-mycophenalate mofetil-prednisone to tacrolimus-mycophenalate mofetil-prednisone at Mayo Clinic, Rochester between April 2001 and January 2006 were reviewed. Of the total of 116 ADPKD patients, 16 patients met the following criteria: **[1]** PLD evident on imagine studies; **[2]** on sirolimus-containing or calcineurin inhibitor based immunosuppression initiated at the time of renal transplantation and not switched from one group to the other during the period of observation; **[3]** on the same immunosuppressive for greater than 6 months; **[4]** abdominal imaging study (CT or MRI) obtained within 12 months prior to the renal transplantation and, for the patients on sirolimus-containing regimen, the repeated imaging while on sirolimus or within 11 months after the termination of sirolimus (in cases where sirolimus was terminated); and **[5]** absence of cyst reductive procedures or liver transplant. Seven patients on sirolimus-containing and 9 on non-sirolimus regimen met these criteria and were included. Their characteristics are summarized in **Table 1**. Laboratory parameters at the times of the first and second scans are summarized in **Table 2**.

Volumetric Determination of the Liver and Native Kidney

The total volumes of the cystic livers and kidneys were measured by a nephrologist (QQ) and a radiologist (BFK) blinded to the patient's immunosuppressive regimens. Single breath-hold CT scans, which demonstrated negligible artifacts from respiration, were used for 29 of the 32 acquisitions with or without intravenous contrast. The remaining 3 were obtained with MRI. CT scanning parameters were 120 kVp, 380 mA, 512 x 512 acquisition matrix, 5 to7 mm collimation, 1 pitch, and 5-7 mm increment with no overlap. MRI parameters were repetition time 2150 ms, echo time 30.0 ms, nex 1.0, 256\128 acquisition matrix, 8 or 15 mm slice thickness. Digital images were reviewed on PC workstation. The cross sectional areas of each cut-slice were measured using a standard software

system (QREADS) at Mayo Clinic, Rochester. Each cross-sectional area was outlined and the software generated a numeric number of the area (in mm^2). The volume of each cut-slice (in mm^3) was calculated by multiplying the cross-sectional area to the thickness of the slice. Two separate measurements were performed. The variation was minimal, and the average was used for analysis.

Immunohistochemistry

Five-micrometer sections from formalin-fixed, paraffin-embedded blocks were deparaffinized in xylene and hydrated with 100%, 95%, 70% ethanol serially; then the sections were exposed to 1.5% H_2O_2 (Sigma-Aldrich) to quench endogenous peroxidases (x 30min) and incubated in proteinase K (Chemicon) (x 30min, 37°C) for antigen retrieval. Non-specific binding was blocked by normal (Rabbit) blocking serum (Vector laboratories). The sections were incubated with rabbit anti-phospho-mTOR (Ser2448) (Abcam), anti-phospho-Akt (Ser473), anti-phospho-ERK (Thr202/Tyr204), and anti-phospho-S6rp (Ser240/244) antibody (Cell Signaling Technology) in normal blocking serum (overnight at RT) in a humidified chamber. The sections were then incubated with appropriate biotinylated secondary antibody and with VECTASTAIN elite ABC reagent (Vector laboratories). To enhance nuclear detail, all slides were counterstained with hematoxylin and then mounted with mounting media (Fisher Scientific).

Statistical Analysis

The numerical values of the liver and kidney volumes for each patient were compared. The data from sirolimus and non-sirolimus groups were compared using Student's *t*-test. The Results were expressed as mean ± SE. A *P* value of <0.05 was considered significant.

Results:

Clinical and laboratory characteristics of renal transplant ADPKD patients with or without receiving sirolimus-containing immunosuppression

Sixteen patients, 7 on a sirolimus-containing (+ sirolimus) and 9 on a tacrolimus-containing (- sirolimus) regimen, met the inclusion criteria. Diagnoses of ADPKD were made based on family history and clinical criteria**(27).** As shown in **Table 1**, with the exception of gender, patients in the sirolimus and tacrolimus groups had a comparable demographical, clinical, and laboratory characteristics at the time of the first imaging study. None of the female patients were on oral contraceptive or hormone replacement therapy. Total liver volumes in the two groups at the time of the first imaging were not significantly different (3.06 ± 0.47 vs. 2.83 ± 0.80, $P = 0.80$).

The adverse effects of sirolimus immunosuppression on bone marrow elements and serum lipids were examined. At the time of the second imaging study, 5 of the 7 patients (71%) in the sirolimus group were on 1 or 2 agents (4 and 1 patients, respectively) for dyslipidemia compared to 5 of the 9 (56%) in the tacrolimus group, all on single agents. As shown in **Table 2**, low density lipoprotein (LDL) level in the sirolimus group was significantly higher than that in non-sirolimus group (122.6 ± 19.6 vs. 73.1 ± 6.7, $P = 0.048$). Average serum triglyceride level was also higher (220.3 ± 40.0 vs. 193.2 ± 30.9, $P = 0.06$). Likewise, platelet counts tended to be lower (154.3 ± 19.9 vs. 210.6 ± 44.0, P = 0.27) and total cholesterol concentrations higher (200.9 ± 17.5 vs. 168.5 ± 6.1, $P = 0.12$) with sirolimus-containing immunosuppression.

Sirolimus-containing immunosuppression is associated with a reduction in PLD liver volume

At the time of the first imaging study, total liver volumes ranged from 1148 to 9072 mL (**Table 1**). Five of the 7 patients in the sirolimus group and 6 of the 9 patients in the tacrolimus group had total liver volumes greater than 2000 mL. As shown in **Figure 1A**, the sirolimus group showed a decrease (-11.85% ± 0.03), while the tacrolimus group showed an increase (+14.13 ± 0.09, $P = 0.009$) in total

liver volume. A suggestive, but not statistically significant correlation between the duration of sirolimus exposure and reduction in liver volume was observed (r = -0.452, $P = 0.12$, **Figure 1B**). The changes in liver volume for each individual patient are shown in **Figure 2**. Representative series of transaxial CT scans from an ADPKD patient before and after 15.5 months of sirolimus-containing immunosuppression (**Figure 3**) illustrate the reduction in the size and number of liver cysts.

Sirolimus-containing immune suppression is associated with a trend towards a greater reduction in native polycystic kidney volume

Shillingford JM et al have shown that sirolimus-containing immunosuppression, given to ADPKD patients after renal transplantation, is associated with a shrinkage of the native cystic kidney volume**(9)**. The renal volumes of 6 patients on sirolimus (1 patient had bilateral nephrectomy, 1 had right-sided nephrectomy) and 7 on non-sirolimus (2 had bilateral nephrectomy, 1 had left-sided nephrectomy) were measured. As shown in **Figure 4**, the average renal volume in the sirolimus group was reduced by 14.76 ± 0.08% and 15.03 ± 0.08% vs. 10.9 ± 0.06% and 9.0 ± 0.06%, right and left kidneys, respectively, in the non-sirolimus group. This trend did not reach a statistical significance, $P =$ 0.38 and 0.28 for right and left kidneys, respectively, possibly due to the small number of patients.

PLD cyst-lining epithelia show an elevated mTOR signaling

We examined the phosphorylated, activated form of mTOR in the liver sections from two ADPKD patients and two non-ADPKD controls. As shown in **Figure 5**, compared to normal biliary epithelia and non-cystic areas of PLD, the cyst-lining epithelia exhibits intense cytoplasmic staining of active, phospho-mTOR. Consistent with this finding, phospho-S6rp, a downstream mTOR effector, was also activated in the cyst epithelium (**Figure 6E-F**). The activation of mTOR signaling may occur downstream to the activation of ERK and AKT, both are activated in PLD (**Figure 6G-J**).

Discussion:

This study demonstrates that a sirolimus-containing immunosuppressive regimen is associated with a reduction in polycystic liver volume, presumably through inhibition of mTOR. Consistent with this interpretation, we show that PLD cyst-lining epithelia express high levels of activated mTOR and its downstream effector, p-S6rp.

mTOR is a serine/threonine protein kinase, activated by a Ras-related GTPase, Rheb (Ras homolog enriched in the brain). Rheb is deactivated by a Rheb-GTPase activating protein (RhebGAP) function of the tuberin-hamartin complex**(28).** Tuberin is known to be phosphorylated by ERK and AKT at multiple sites, with a resultant dissociation from the tuberin-hamartin complex. This dissociation interrupts the tuberin-hamartin complex-mediated Rheb inhibition, leading to an augmented mTOR signaling**(29, 30)**. The finding of highly activated ERK and AKT in the PLD cyst-lining epithelium (**Figure 6**, **G-J**) is consistent with the possibility of ERK/AKT-mediated mTOR activation.

mTOR forms two distinct protein complexes within mammalian cells: regulatory-associated protein of TOR (raptor) and rapamycin-insensitive companion of TOR (rictor)**(31, 32)**. mTOR-raptor complex, inhibited by sirolimus, promotes cell growth by at least two mechanisms. First, mTOR-raptor complex phosphorylates the S6 ribosomal protein (S6rp) kinase and S6rp, thereby augmenting protein translation and ribosomal biogenesis**(33)**. Second, it inactivates (by phosphorylation) the eukaryotic initiating factor 4E (eIF4E)-binding protein (4E-BP1), dissociating 4E-BP1 from the RNA cap-binding protein eIF4E, promoting cap-dependent mRNA translation**(34)**. Sirolimus has been shown to bind the FKBP-rapamycin-binding domain of mTOR. This binding destabilizes the association between mTOR and raptor, preventing the downstream phosphorylations of S6rp kinase/S6rp and 4E-BP1**(35)**.

Although previous studies suggest that mTOR-rictor complex is insensitive to sirolimus, recent reports raise the possibility that sirolimus may also affect mTOR-rictor signaling. mTOR-rictor complex modulates cellular proliferation by phosphorylating the survival factor AKT at Ser 473**(36, 37)**. In breast cancer and anaplastic-large-cell-lymphoma cells, sirolimus treatment reduces the number of viable cells and promotes chemotherapy-induced apoptosis. These effects are associated with reductions in both phospho-mTOR (Ser2448p-mTOR) and phospho-AKT (Ser473p-AKT), possibly via a direct and indirect mechanisms**(38-41)**.

The role of aberrant mTOR activation in promoting cystic liver enlargement is supported by the observation of a uniform reduction in liver volume in patients receiving sirolimus-containing regimen. Since female ADPKD patients tend to have more progressive PLD growth associated with estrogen exposure, the higher number of females in the non-sirolimus group might potentially have contributed to the difference detected between the two groups. However, this is unlikely because male patients, rather than female patients, in the non-sirolimus group had the most pronounced liver growth. As shown in **Figure 2B**, the two patients with the largest percentage liver volume increase were males. One female patient with a liver volume of 9.07 L prior to renal transplantation actually showed a reduction, possibly due to a nearly maximal degree of abdominal distension. Furthermore, a uniform liver volume reduction, observed in the sirolimus group (**Figure 2A**), would be highly atypical and defy the natural course of PLD. Taken together, the gender distribution could not explain the observations in this study.

Another potential explanation for these results is that the detected difference is related to the avoidance of calcinurin inhibitors rather than the exposure to sirolimus. Indeed, one of the functions of PC1 is to activate calcineurin/NFAT (nuclear factor of activated T-cells) signaling**(42).** The use of calcineurin inhibitor (cyclosporine) has been associated with a higher frequency of acquired cystic

kidney diseases following renal transplantation**(43).** However, because PC1-mediated calcineurin/NFAT activation is presumably disrupted in ADPKD, further inhibition with calcineurin inhibitor may exert lesser effect. The consistent reduction in liver volume with sirolimus also renders this explanation unlikely.

We have examined the volumes of native polycystic kidneys following renal transplantation in our patients. Consistent with a previous report**(9)**, the reduction in the native kidney volume tends to be more pronounced in the patients treated with sirolimus. However, the difference did not reach a statistical significance (**Figure 4**). This could possibly result from the small number of patients. It is also possible that sirolimus given orally is more efficacious to treat polycystic liver than polycystic kidney disease. Sirolimus is absorbed in the small intestine and undergoes an extensive presystemic metabolism by the intestinal and hepatic cytochrome P-450 system (CYP3A4), followed by biliary excretion of its metabolites. At least two of the six identified metabolites have retained immunosuppressive activity**(44, 45)**. This presystemic biliary exposure to sirolimus/its active metabolites, combined with a reduced drug delivery to end-stage polycystic kidneys due to a compromised renal blood flow, may in part account for the observed differential efficacy of sirolimus in the polycystic liver and kidneys.

Because liver volume, not derangement of liver function, is the major source of morbidity and mortality in PLD, the observation of a significant reduction in polycystic liver volume with sirolimus-containing regimen is encouraging. Although a prospective, confirmatory study is necessary, sirolimus shows promise as a potential treatment option for severe PLD.

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LEGEND:

Table 1. The patients' characteristics at the time of the first abdominal image, the interval between the two images, and duration of the sirolimus treatment.

Table 2. Laboratory parameters before and after the sirolimus-containing and sirolimus-sparing immune suppression

Figure 1

A. Average changes in the liver volume in ADPKD patients with or without receiving sirolimus.

B. The reduction in total liver volume plotted as a function of the duration of sirolimus treatment.

Figure 2. Total liver volume in each individual patient at the 1st and 2nd imaging studies. Each circle or square represents a single subject. Circle = female, Square = male.

Figure 3. Representative series of transaxial CT sections obtained from an ADPKD patient at the mid-level of the liver before (left column) and after (right column) 15.5 months of the sirolimus-containing immune-suppressant, showing a reduction in the size and number of liver cysts. The top cuts of the 2 CT series are aligned at the center of a partially calcified cyst.

Figure 4.

The changes of the native polycystic kidneys at the 1st and 2nd imaging studies

A and **C** show average changes, mean and SE, of right and left kidney volumes in patients with or without receiving the sirolimus-containing immunosuppression. **B** and **D** show the total right and left kidney volumes in each individual patient at the time of the 1st and 2nd imaging studies.

Figure 5.

Phospho-mTOR is elevated in PLD cyst-lining epithelia. Liver sections from two normal subjects (**A** and **B**) and two PLD patients (**C**-**F**) were immunostained with antibody against Ser2448 phospho-mTOR. PLD cyst-lining epithelial cells from both ADPKD patients showed intense staining for phospho-mTOR (**C** and **D**, enlarged views in **G**-**I**). The phospho-mTOR was non-detectable in the biliary epithelia from normal controls (**A** and **B**) and almost non-detectable in the non-cystic biliary epithelia at portal triads (**E** and **F**) of the same PLD sections as in **C** and **D**.

Figure 6.

The effectors of phospho-mTOR, phospho-S6 ribosomal protein and phospho-AKT are elevated in PLD cyst-lining epithelia.

Liver sections from a normal subject were immunostained with antibodies specifically recognize mTOR downstream effector, p-S6rp (**A**), and p-AKT (**B**). Consecutive PLD sections from an ADPKD patient (**C**, **E**, **G**, and **I**) were immunostained with antibodies against Ser2448 p-mTOR (**C**), Ser240/244 p-S6rp (**E**), Ser473 p-AKT (**G**), and Thr202/Tyr204 p-ERK (**I**). **D**, **F**, **H**, and **J** are the enlarged views of **C**, **E**, **G**, and **I**, respectively. PLD cyst-lining epithelia show a high level of staining for activated mTOR, S6rp, AKT, and ERK, while the normal biliary epithelia show non-detectable p-S6rp (**A**) and p-AKT (**B**).

Mean (range)	+ Sirolimus, no.7	- Sirolimus, no.9
Age, year	55.4 (41-66)	57.6 (48-67)
Gender, Female/Male	2/5	6/3
Systolic blood pressure, mmHg	134.3 (88-180)	128.1 (121-155)
Diastolic blood pressure, mmHg	80.9 (55-90)	76.3 (66-97)
Total liver size, L	3.06 (1.71-5.35)	2.83 (1.15-9.07)
Duration between 2 scans, months	30 (11-58)	28.7 (17-46)
Duration of sirolimus treatment, months	19.4 (7-40)	n/a
Serum sirolimus concentration, ng/mL	14.3 (7.8-17.9)	n/a

Table 1. The patients characteristics at the time of the first abdominal image, the interval between the 2 imagines, and duration of the sirolimus treatment.

Table 2. Laboratory parameters before and after the sirolimus-containing and sirolimus-sparing-immune suppression

Mean (range)	+ Sirolimus 1st scan	+ Sirolimus 2nd scan	- Sirolimus 1st scan	- Sirolimus 2nd scan
HGB, g/dL	11.8 (9.4-15.4)	13.3 (11.8-16.8)	12.9 (10.1-14.4)	12.6 (9.7-14.7)
Platelet, 10^9/L	172.1 (151-214)	154.3 (76-249)	200 (89-338)	210.6 (65-523)
Serum creatinine, mg/dL	3.59 (2.3-9.7)	1.46 (0.8-2.4)	5.86 (1.3-7.5)	1.48 (1.1-2.3)
Total Cholesterol, mg/dL	173.9 (149-195)	200.9 (128-265)	196.7 (121-337)	168.5 (145-204)
LDL, mg/dL	101.6 (80-135)	*122.6 (81-209)	90.8 (60-117)	*73.1 (41-105)
HDL, mg/dL	43.9 (29-87)	46.7 (12-70)	48.3 (35-60)	51.6 (25-77)
Triglyceride, mg/dL	168.9 (53-441)	220.3 (127-434)	264.2 (55-687)	193.2 (102-392)
Albumin g/dL	4.1 (3.7-4.5)	3.9 (3.4-4.4)	3.9 (3.3-4.8)	4.1(3.7-4.3)

* indicates a statistically significant difference between + sirolimus group and – sirolimus group at the time of the second scan.

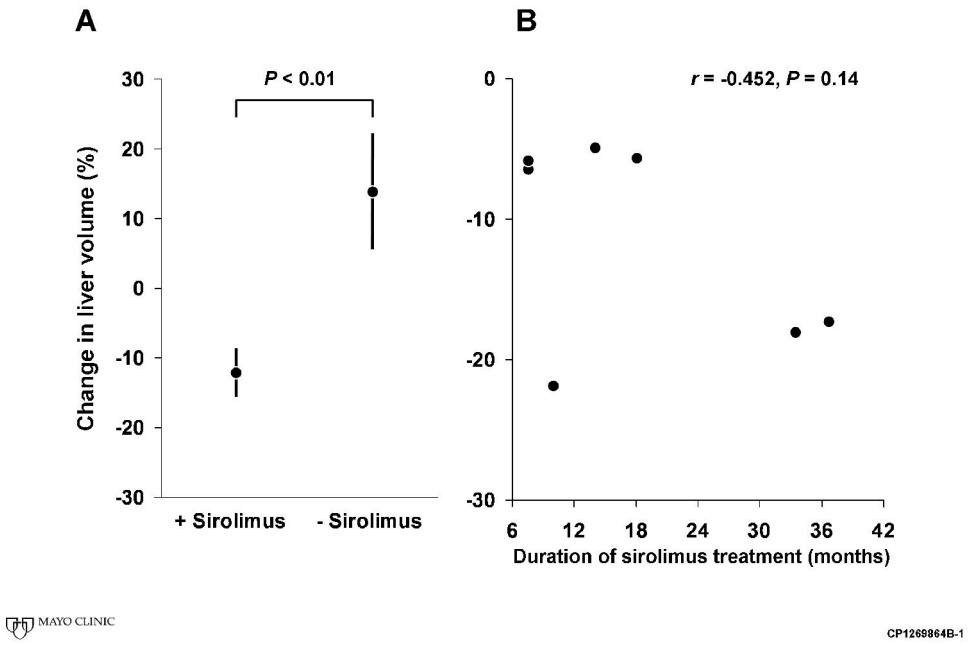


Figure 1 A. Average changes in the liver volume in ADPKD patients with or without receiving sirolimus. B. The reduction in total liver volume plotted as a function of the duration of sirolimus treatment.
254x190mm (300 x 300 DPI)

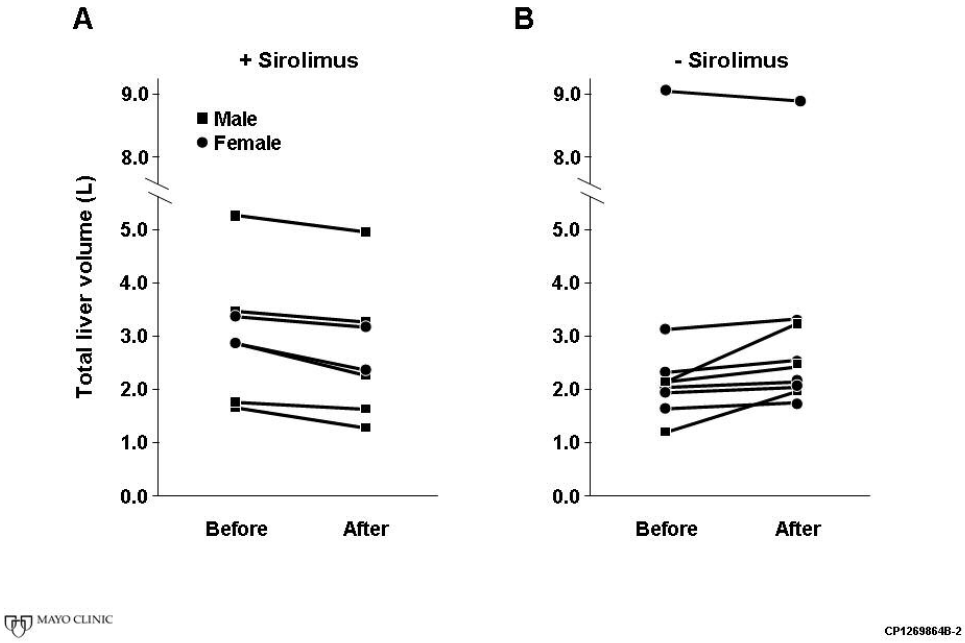
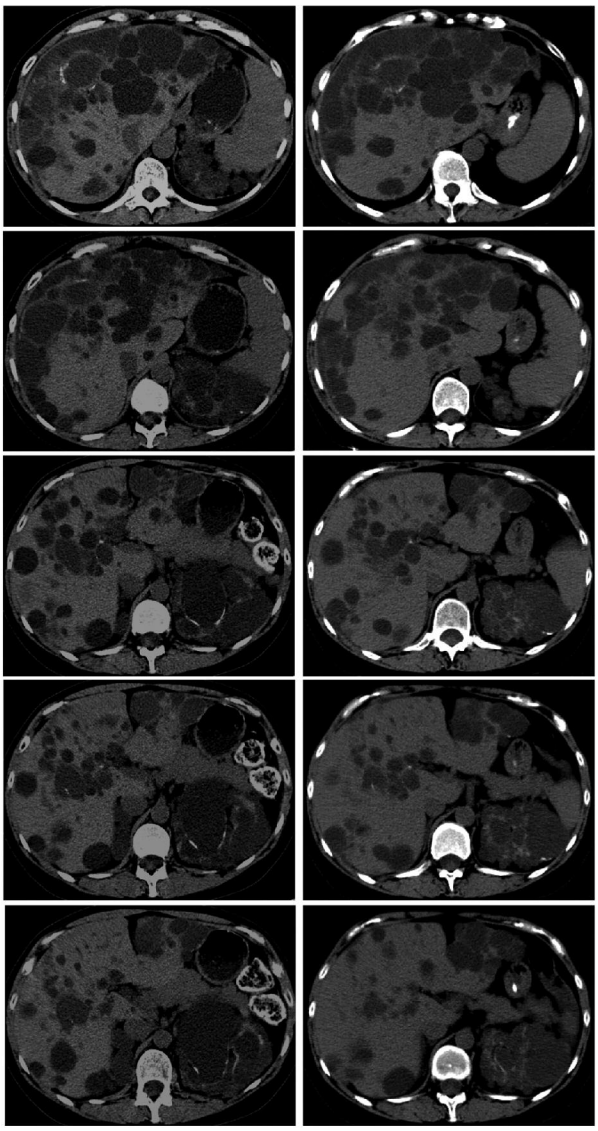


Figure 2. Total liver volume in each individual patient at the 1st and 2nd imaging studies. Each circle or square represents a single subject. Circle = female, Square = male.
254x190mm (96 x 96 DPI)



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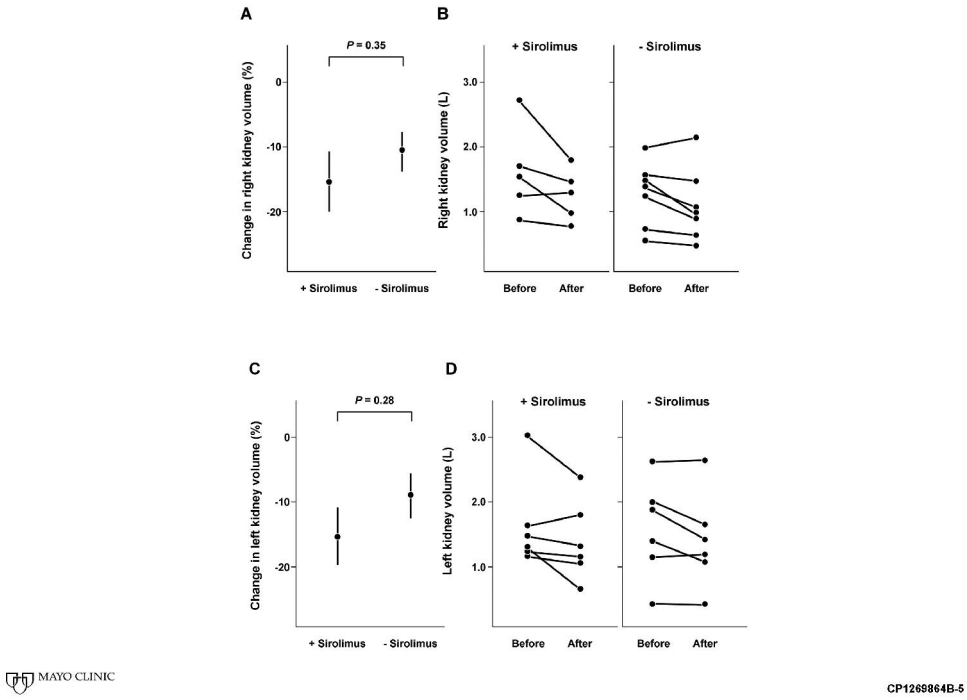
116x203mm (300 x 300 DPI)



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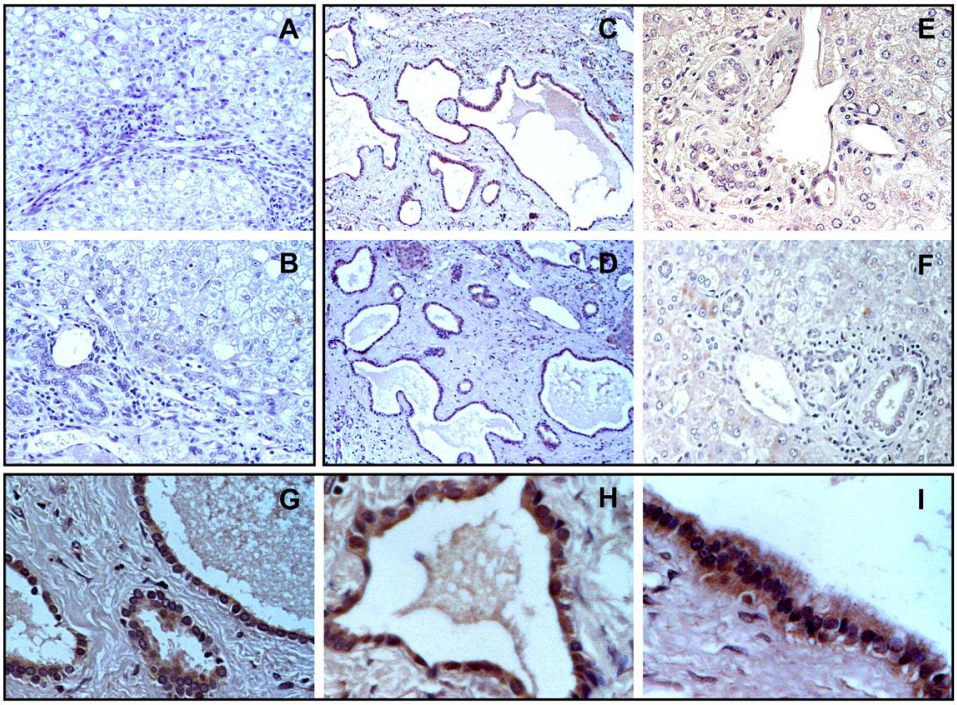
254x190mm (300 x 300 DPI)



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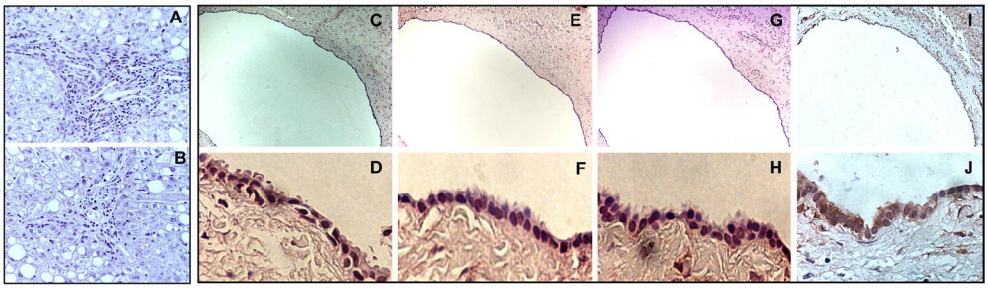
203x151mm (150 x 150 DPI)



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203x60mm (200 x 200 DPI)