



Original Articles

Longitudinal Effects of Everolimus on White Matter Diffusion in Tuberous Sclerosis Complex



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ABSTRACT

OBJECTIVE: We studied the longitudinal effects of everolimus, an inhibitor of the mammalian target of rapamycin (mTOR), on callosal white matter diffusion tensor imaging (DTI) in patients with tuberous sclerosis complex (TSC).

METHODS: Serial imaging data spanning nine years were used from the open label, Phase I/II trial (NCT00411619) and open-ended extension phase of everolimus for the treatment of subependymal giant cell astrocytoma associated with TSC. From 28 patients treated with everolimus and 25 untreated control patients, 481 MRI scans were available. Rigorous quality control resulted in omission of all scans with diffusion weighted imaging data in less than 15 directions or more than eight artifacted volumes, and all postsurgical scans. We applied a linear mixed-effects model to the remaining 125 scans (17 treated, 24 controls) for longitudinal analysis of each DTI metric of manually drawn callosal regions of interest.

RESULTS: On a population level, mTOR inhibition was associated with a decrease in mean diffusivity. In addition, in treated patients only, a decrease of radial diffusivity was observed; in untreated patients only, an increase of axial diffusivity was seen. In patients below age 10, effect-sizes were consistently greater, and longer treatment was associated with greater rate of diffusion change. There was no correlation between DTI metrics and reduction of subependymal giant cell astrocytoma volume, or everolimus serum levels.

CONCLUSIONS: Effects from mTOR overactivity on white matter microstructural integrity in TSC were modified through pharmacologic inhibition of mTOR. These changes sustained over time, were greater with longer treatment and in younger patients during a time of rapid white matter maturation.

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Conflicts of interest: D.F. and D.K. led the original Phase I/II trial (NCT00411619) and the open-ended extension phase, from which these data were derived, funded by Novartis. J.P., M.S., D.F., and D.K. have participated in the Novartis EXIST-III trial (NCT01713946), although these data were not used for this study. D.K. has received consulting and speaking fees and travel expenses from Novartis and Upsher-Smith Pharmaceuticals. In addition, he has received research support from Novartis Pharmaceuticals. D.F. discloses research support and a consulting role with Novartis, and a consulting role with Aeonian Pharmaceuticals. MS reports grant support from Novartis, Roche, Pfizer, Ipsen, LAM Therapeutics, and Quandrant Biosciences. He has served on Scientific Advisory Boards for Sage, Roche, and Takeda. Novartis did not provide funding of any sort, or editorial assistance of any kind, to this work. In addition, all clinical data and relevant images were available to the authors for review and processing.

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Introduction

Tuberous sclerosis complex (TSC) is a genetic autosomal dominant neurocutaneous disorder related to pathogenic variants in the *TSC1/TSC2* genes with subsequent overactivity of the mechanistic target of rapamycin (mTOR) pathway. Neurological symptoms include epilepsy, intellectual disability, behavioral dysregulation, autism spectrum disorder, and growth of deep, potentially obstructive lesions called subependymal giant cell astrocytoma (SEGA). The clinical phenotype is highly variable and unpredictable, and worse neurodevelopmental outcomes are seen with early and severe epilepsy.¹

Biomarkers are needed to identify high-risk patients, for clinical trials of early and preemptive seizure control and for mitigation of early autistic symptoms through intense behavioral programs.^{2,3} A biomarker of neurological disease severity in TSC should be accurate and reproducible, and ideally reflect the biologic process, pathogenic process, or therapeutic intervention.

Diffusion tensor imaging (DTI) of the white matter is potentially such a biomarker, as DTI metrics of various tracts correlate with autism spectrum disorder, refractory epilepsy, and intellectual disability.⁴ Microstructural integrity of the corpus callosum reflects the overall neurological disease burden but is not specific to any symptom. mTOR inhibitors dampen the overactive mTOR pathway, two steps downstream from the malfunctioning *TSC1/2* protein complex in TSC. Everolimus (Afinitor/Votubia, Novartis, East Hanover, NJ) is FDA-approved for treatment of renal angiomyolipomas, SEGA, and recently for the adjunctive treatment of refractory seizures in TSC.⁵ The Phase I/II trial demonstrated a significant reduction in SEGA volume, and the long-term efficacy and safety data of this same cohort exhibited a sustained effect with continuing reduction in SEGA size.⁶

In addition to SEGA volume reduction, we previously reported altered DTI measurements after 12 to 18 months on treatment. In a group of 21 treated patients, fractional anisotropy (FA) increased and mean diffusivity (MD) decreased in the corpus callosum and other regions of interest (ROIs), while no such change was seen in a group of age- and gender-matched controls with TSC but no treatment.⁷

In a recent Phase III trial, longer exposure to everolimus was associated with an incremental effect on seizure reduction,⁵ and a better response was seen with younger age.⁸ We hypothesized that treatment effects on white matter DTI metrics would also be sustained over time. To test this hypothesis, we expanded on our previous work and analyzed the longitudinal diffusion imaging data from the same patients in the Phase I/II trial and open-ended extension phase.^{6, 7} In addition, we examined effects from age, duration of treatment, everolimus serum levels, and percent SEGA volume reduction.

Methods

Study design

This study included participants enrolled in an open label, Phase I/II trial (NCT00411619) and open-ended extension phase of

everolimus for the treatment of SEGAs associated with TSC, conducted at Cincinnati Children's Hospital Medical Center. Twenty-eight TSC patients older than age three were enrolled. All patients were diagnosed following clinical and/or genetic criteria of TSC, and had serial growth of SEGA on two or more consecutive MRIs. Treatment protocol, serum, and MRI monitoring has been reported previously.^{9,10} Target everolimus concentrations were 5 to 15 ng/mL. SEGA and hydrocephalus were monitored with volumetric MRI at three, six, and 12 months after initiation of everolimus, and every six months thereafter unless clinical course dictated otherwise.

The control group included imaging data from 25 age- and gender-matched patients with TSC, identified through a database from the CCHMC TSC clinic. Participants met clinical and/or genetic criteria of TSC and had no history of exposure to medication affecting the mTOR pathway. Scans were obtained per TSC clinical surveillance guidelines^{11,12} and not part of the Phase I/II trial; thus, scan intervals were not as frequent but the acquisition protocol was similar.

Informed consent was obtained from adult patients and from parents or guardians for patient under 18 years of age. Assent was provided by patients when able per institutional requirements. The CCHMC institutional review board approved the protocol.

MRI acquisition

MRI scans were acquired on 1.5T GE Signa HDxt, 1.5T GE Signa Excite, 3T GE Discovery, 3T Philips Achieva, and 3T Philips Ingenia scanners over a course of nine years (May 2006 to June 2015). The imaging protocol included: sagittal MPRAGE, T2w TSE, and 15 diffusion-weighted echoplanar images acquired in the axial plane (b-value 1000 s/mm², repetition time/ echo time 12,000/81 to 101, field of view 384 × 384 mm, 3 mm³) covering the entire brain. Note that diffusion weighted imaging (DWI) was not part of the formal Phase I/II study protocol, and that during the course of the open-ended extension phase, MRI scanners underwent upgrades and scans were performed at local hospitals and transmitted to CCHMC, resulting in some MR protocol variability and warranting extensive quality control procedures (see below).

MRI quality assurance

Four hundred eighty-one scans from 28 patients and 25 controls were available for analysis. T1w, T2w, and all DWI volumes were reviewed for artifact (motion, susceptibility, and scanner-related) by expert raters. Scans were excluded as follows: no DWI acquired (n=54), fewer than 15 DWI directions acquired (n=68), greater than eight DWI volumes of artifact due to patient motion, magnetic susceptibility, severe echo planar imaging distortion, or hardware-induced artifact (n=181), and prior neurosurgery (n=53) (Fig 1).

After curation of the MRI dataset, 17 patients on everolimus were included with 76 MRIs on three different scanners: 3T Phillips Achieva (n=8), 1.5T GE Excite (n=7), and 1.5 GE Signa HDxt (n=61). Twenty-four control patients had 49 MRIs of sufficient quality, acquired on five different scanners: 3T Phillips Achieva (n=3), 1.5T GE Excite (n=8), 1.5 GE Signa HDxt (n=36), 3T GE Discovery MR750w (n=1), and 1.5T Phillips Ingenia (n=1) (Table 1).

MRI processing

All MRI processing and analyses were performed using the Computational Radiology Kit (<http://crl.med.harvard.edu>). For each scan, the T2w was aligned to the T1w image and the intracranial cavity was segmented using a previously validated multispectral method.¹³ DWI data were screened for intravolume motion and scanner artifact by expert raters, and artifacted volumes were removed. Intervolume motion correction was performed via affine registration of each DWI volume to the mean b=0 s/mm² image. DWI was upsampled to the T1w image and skull stripped with the intracranial cavity segmentation. A single-tensor diffusion model

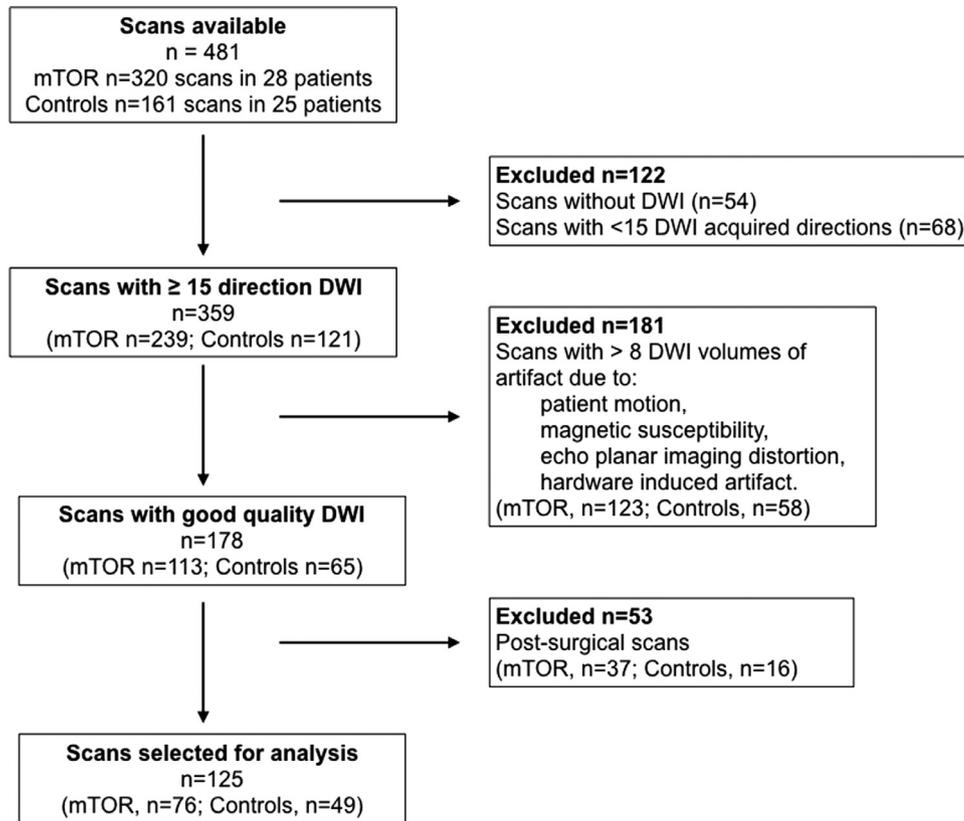


FIGURE 1.

MRI quality assurance and data selection process. Of the 481 scans available for all patients, 125 of 17 patients treated with everolimus and 24 intreated controls with TSC were included in data analysis after rigorous quality assurance. Scans with only some artifacted volumes could often be salvaged through reconstruction of the tensor data after omitting bad volumes. DWI = diffusion-weighted imaging; mTOR = mechanistic target of rapamycin

was estimated using robust least squares in each brain voxel from which fractional anisotropy ($FA = 3\text{Var}(\lambda)/(\lambda^2_1 + \lambda^2_2 + \lambda^2_3)^{1/2}$), mean diffusivity ($MD = (\lambda_1 + \lambda_2 + \lambda_3)/3$), radial diffusivity ($RD = (\lambda_2 + \lambda_3)/2$), and axial diffusivity (λ_1) were computed.

Callosal ROIs were manually drawn in five parasagittal planes in each scan, with ITK-SNAP 2.0 (www.itksnap.org). Mean values of FA, MD, RD, and AD were measured in each ROI.

Statistical analysis

Statistical analyses were performed using software R (CRAN, Vienna, Austria, www.R-project.org) and SAS Version 9.4 (SAS Institute, Inc, Cary, NC). Longitudinal analysis of each DTI metric for the callosal ROI was done using a linear mixed-effects model. The model consisted of intercept, slope of log age at baseline, slope of within-subject (WS) effect of log age, and interaction of treatment status (everolimus versus controls) with the WS effect of log age as fixed effects. The random intercept and slope terms were included to account for WS correlations and between subject variability.

Likelihood ratio tests were used to determine the significance of variance covariance parameters of the random-effects among nested models. Final model selection was performed using Akaike Information Criterion. All fixed-effects were retained in the final models irrespective of their statistical significance to understand the relationship between DTI metrics and treatment status. For the analysis, DTI FA was multiplied by a scale factor of 10, and DTI MD, RD, and AD were multiplied scaled by a scale factor of 1000. Finally, missing data were not imputed and all the available measurements were included in the analysis under the missing-at-random assumption.

Given the steep developmental trajectory of diffusion metrics before approximately age 10,^{14,15} and a higher response rates of seizures in younger patients,⁸ we analyzed the data separately for those less than 10 years of age. Duration of treatment (at time of each scan), mean everolimus levels (over the full course of treatment), and maximum percentage of SEGA volume decrease were introduced in the model as continuous variables.

Results

Patients

Demographic and clinical data of included patients are shown in Table 1. While the Phase I/II trial and open-ended extension phase contained 28 patients, and we previously reported on diffusion imaging findings in 21 of these,⁷ in the current work the quality of the data limited the analysis to 17 patients. There were more males in the treated group, and age was higher in the untreated group. The total number of scans per patient and interval between scans was higher in the treated group. Mean duration of treatment was 3.5 years. The mean serum level (5.8 ng/mL), and mean maximum SEGA volume decrease (50.8%) showed little variation for the length of the study across the majority of patients receiving treatment.

TABLE 1.
Patient Demographics, MRI Data

| | ≤10 years | | All | |
|--|--------------|-------------|-------------|-------------|
| | mTOR | Controls | mTOR | Controls |
| Number of participants | 8 | 14 | 17 | 24 |
| Sex (%male) | 75 | 50 | 71 | 54 |
| Age, mean (SD) (years) | 9.1 (1.8) | 7.8 (2.2) | 12.8 (4.7) | 14.7 (9.0) |
| Age range (years) | [3.5, 10.87] | [3.7, 10.7] | [3.5, 25.7] | [3.7, 44.3] |
| Duration of treatment, mean (SD) (years) | 3.4 (1.6) | – | 3.5 (2.3) | – |
| Serum levels, mean (SD) (ng/mL) | 5.7 (1.9) | – | 5.8 (2.6) | – |
| % Maximum SEGA volume decrease, mean (SD) (SD)(SD) | 50.8 (20.1) | – | 50.8 (19.6) | – |
| Number of scans | 31 | 18 | 76 | 49 |
| Number of scans per participant, mean (SD) | 4.1 (2.2) | 1.3 (0.6) | 4.5 (3.0) | 2.0 (1.04) |
| Number of DWI volumes removed, mean (SD) | 0.4 (1.2) | 0.2 (0.7) | 0.3 (0.9) | 0.4 (1.3) |
| Number of participants with 1 scan | 1 (12.5%) | 11 (79%) | 5 (29%) | 7 (29%) |
| Number of participants with 2 scans | 1 (12.5%) | 2 (14%) | 1 (6%) | 13 (55%) |
| Number of participants with 3 scans | 1 (12.5%) | 1 (7%) | 2 (12%) | 1 (4%) |
| Number of participants with 4 scans | 1 (12.5%) | 0 | 0 | 2 (8%) |
| Number of participants with 5 or more | 4 (50%) | 0 | 9 (53%) | 1 (4%) |

Abbreviations:
 DWI = diffusion weighted imaging
 SEGA = subependymal giant cell astrocytoma
 mTOR patients on everolimus, an inhibitor of the mechanistic target of rapamycin.

DTI

Table 2 summarizes the findings in our model, and Fig 2 displays longitudinal changes in the subgroup of patients below 10 years of age. Of importance, the model assesses for mean changes on a population level, compared with controls.

In all ages combined, and in the subgroup below 10 years of age, FA increased in both treated and untreated patients. This age-related FA increase occurred at the same rate in treated and untreated patients.

Across all ages, and in the younger patients, no changes in MD with age were seen in the untreated patients. The MD decreased, however, in the treated patients across all ages, and in the younger subgroup. As a result, this longitudinal MD change was significantly different between treated versus untreated patients.

In all patients, the RD decreased only in the treated patients; this was also seen in the subgroup of patients

below 10 years old. The RD in the untreated patients did not significantly change. The RD changes, however, were not different between treated versus untreated patients.

In all patients, as well in the younger subgroup, the AD in the untreated patients increased. It did not change in treated patients. For all patients and in the younger patient subgroup, the AD changes over time were significantly different in treated versus untreated subjects.

Treatment variables

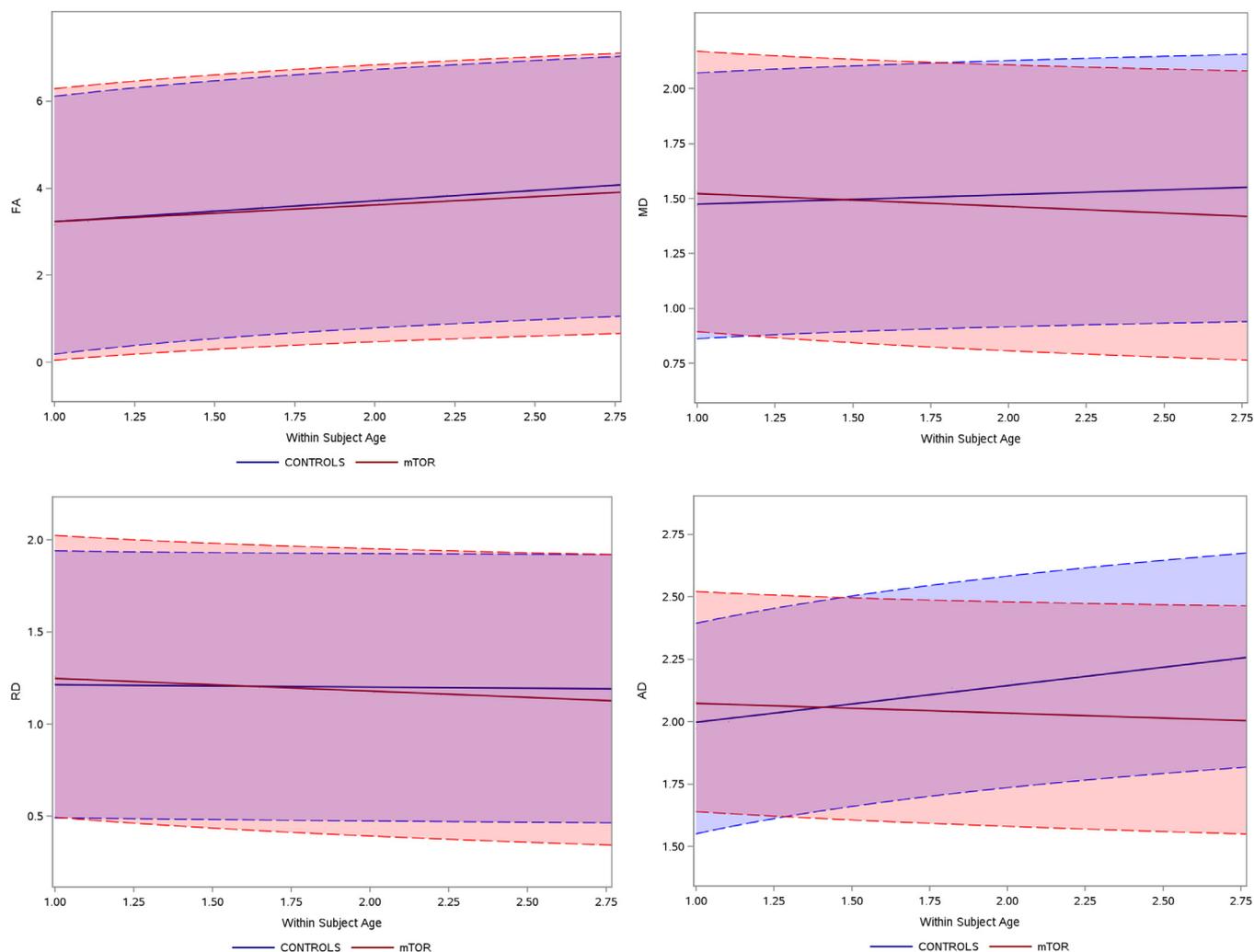
With respect to (log) age, the linear rate of FA change was not associated with duration of everolimus treatment when evaluated for all patients. In the younger subgroup, however, steeper increases were seen with longer treatment ($P = 0.024$).

For MD and RD a similar but inverse association was seen between rate of change and duration of treatment ($P = 0.0321$ and $P = 0.014$, respectively). This association

TABLE 2.
Longitudinal Effects on Diffusion Metrics in Treated and Untreated Patients with Tuberous Sclerosis Complex

| | Within subject age effect | ≤ 10 years | | | All | | |
|----|----------------------------|------------|-------------------------|---------|----------|-------------------------|---------|
| | | Estimate | 95% Confidence interval | P value | Estimate | 95% Confidence interval | P value |
| FA | Controls | 0.8797 | (0.368, 1.392) | 0.0012 | 0.647 | (0.181,1.114) | 0.007 |
| | Patients on mTOR-inhibitor | 0.7037 | (0.241, 1.167) | 0.0038 | 0.654 | (0.210,1.0987) | 0.0044 |
| | mTOR versus controls | -0.1759 | (-0.866, 0.514) | 0.61 | 0.00682 | (-0.634,0.650) | 0.983 |
| MD | Controls | 0.0799 | (-0.00895, 0.169) | 0.0768 | 0.0549 | (-0.0191,0.129) | 0.144 |
| | Patients on mTOR-inhibitor | -0.108 | (-0.188, -0.0277) | 0.0096 | -0.092 | (-0.163,0.0218) | 0.0108 |
| | mTOR versus controls | -0.188 | (-0.307, -0.0681) | 0.0029 | -0.147 | (-0.249,-0.0452) | 0.0052 |
| RD | Controls | -0.0228 | (-0.124, 0.0783) | 0.652 | -0.0177 | (-0.101,0.0659) | 0.675 |
| | Patients on mTOR-inhibitor | -0.126 | (-0.217, -0.0344) | 0.0081 | -0.113 | (-0.193,-0.0335) | 0.0059 |
| | mTOR versus controls | -0.103 | (-0.239, 0.0334) | 0.135 | -0.0953 | (-0.211,0.0200) | 0.104 |
| AD | Controls | 0.270 | (0.162, 0.378) | <.0001 | 0.172 | (0.0706,0.273) | 0.0011 |
| | Patients on mTOR-inhibitor | -0.0722 | (-0.171, 0.0262) | 0.146 | -0.0475 | (-0.145,0.0499) | 0.335 |
| | mTOR versus controls | -0.342 | (-0.488, -0.196) | <.0001 | -0.219 | (-0.359,-0.0796) | 0.0024 |

Abbreviations:
 AD = axial diffusivity
 FA = fractional anisotropy
 MD = mean diffusivity
 mTOR = mechanistic target of rapamycin
 RD = radial diffusivity;

**FIGURE 2.**

Longitudinal diffusion changes, treated (mTOR) versus untreated, ≤ 10 years. Diffusion changes over time in treated group (mTOR, red) and untreated group (controls, blue). Orange and blue shaded areas indicate confidence intervals. On the y-axis, the four different DTI metrics. Note that for the analysis, FA was multiplied by a scale factor of 10, and DTI MD, RD, and AD were multiplied scaled by a factor of 1000. On the x-axis, age is expressed a ratio: For example, if the subject is two years at time-point 1.0, the patient will be five years old at time-point 2.5. AD = axial diffusivity; FA = fractional anisotropy; MD = mean diffusivity; RD = radial diffusivity. (The color version of this figure is available in the online edition.)

was also present in the younger subgroup ($P = 0.039$ and 0.0205 , respectively).

Longitudinal DTI metrics were not statistically associated with the maximum SEGA volume decrease, or with mean serum levels (Supplementary Table 1).

Discussion

We demonstrated that pharmacologic inhibition of mTOR was associated with a decrease in MD which was not seen in untreated patients with TSC. In treated patients only, a decrease of RD was observed; in untreated patients only, an increase of AD was seen. Effect-sizes were consistently greater in the younger sub-population. Through rigorous quality control of heterogeneous longitudinal imaging data, our previous findings confirmed and expanded our earlier study suggesting

genetic effects from mTOR overactivation on the white matter can be modified.⁷

In the EXIST-3 trial of adjunctive everolimus for refractory focal-onset seizures in TSC, further seizure reduction was seen with ongoing treatment⁵ and efficacy was higher in younger patients.⁸ Similarly, in our longitudinal imaging study, diffusion changes were sustained, steeper over time with ongoing treatment and more evident in patients under 10 years of age. A dose-response effect, as seen in the EXIST-3 trial in which everolimus was stratified by design into lower and higher target dosing ranges, could not be established in this study. This is likely due to relatively homogenous everolimus serum levels in our cohort in which all subjects belonged to a single targeted dosing range. We continued to see no association with percent SEGA volume change, as previously reported.⁷

Previously reported lower FA and higher MD in the callosal white matter of patients with TSC as compared with healthy controls were mainly driven by an increase in RD,^{16–18} or in both RD and AD.^{4,15}

For RD, a potential explanation could be qualitative changes in myelination. In the white matter of a neuronal *Tsc1* knockout model of TSC, hypomyelination was rescued by mTOR inhibition.¹⁹ In an oligodendrocyte precursor *Tsc2*-knockout mouse model, diffuse hypomyelination was associated with decreased RD.²⁰ In pediatric patients with TSC, *ex vivo* diffusion imaging of resection specimen from epilepsy surgery revealed consistent correlations between myelin content and MD (unpublished data).

Alternatively, imaging and neuropathological studies have demonstrated extensive heterotopic neurons and cells of mixed glial and neuronal lineage throughout the white matter.^{14,21} A reduction in volume or morphology of such cells through mTOR inhibition could lead to increased axonal packing, and a subsequent lower RD.

The AD increased in untreated patients AD over time, which could be due to an increase in axon diameter, as seen on electron microscopy in *Tsc1* neuronal knockout mice.²² This increase, however, is opposite of a typical age-effect previously reported.¹⁴ No such changes of AD were seen in the treatment group, possibly related to inhibition of mTOR.

Two indirect mechanisms could also be responsible for the observed changes in diffusivity. First, impending hydrocephalus or altered ventricular morphology from elevated cerebrospinal fluid pressure could increase extracellular water in the periventricular white matter. Transependymal flow, however, was not seen on conventional imaging and there was again no correlation of mean diffusion metrics with reduction in SEGA volume. In addition, we have previously demonstrated similar diffusion changes in tracts more remote from the ventricles.⁷

Second, refractory epilepsy as a comorbid condition in TSC is associated with a greater increase in diffusivity,^{4,23} and longitudinal effects on diffusion have also been reported.²⁴ Reduced seizures were seen in the treatment group,¹⁰ potentially resulting in altered diffusivity as compared with untreated patients. Seizure frequency, however, was assessed via parental report (and video-electroencephalography in some) and the study design did not allow for a formal prospective analysis.⁷

Limitations include the low number of subjects and only one experienced collaborator manually marked the ROIs. This work is technically limited by a variable quality of the imaging, warranting a meticulous process of data review, and either salvaging or omitting bad scans. Nonetheless, the data remained noisy with a risk of both type I and type II errors. It is possible that the lack of FA changes is due to decreased robustness of FA as compared with MD with regards to motion artifact,²⁵ and also explains why generation of callosal tractography data was unsatisfactory. The inclusion of poor quality data can severely affect findings; in our study, more MRI volumes needed removal in the treatment group (data on file), potentially biasing results. On the other hand, reliability of DTI data can be improved through implementation of stringent quality control measures.²⁶

In summary, callosal DTI metrics reflect the neurological disease burden in TSC,⁴ and the current work demonstrates that these metrics respond to targeted treatment. To truly study the effects of mTOR inhibitors on white matter diffusion in the context of biomarker discovery, however, a proper multicenter prospective data collection effort is needed. Such a study would include strict acquisition protocols, centralized quality control with periodic calibration of both phantom, and human data across sites.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.pediatrneurol.2018.10.005](https://doi.org/10.1016/j.pediatrneurol.2018.10.005).

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