SUPPLEMENTAL MATERIAL

Rho Kinase Inhibition Blunts Lesion Development and Hemorrhage in Murine Models of Aggressive *Pdcd10/Ccm3* Disease

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Supplemental Methods

Vertebrate Animals

The breeding of mice required for all experiments was conducted at the Duke University site. Mice (*Mus musculus*) with the respective genotypes listed below were used. Mouse lines carrying the knockout allele of *Krit1* were generated and are currently maintained at the Duke site. Mouse lines with the knockout alleles of *Pdcd10* were obtained from Dr. Murat Gunel under Material Transfer Agreement with Yale University and are currently maintained at the Duke site. Mice containing the knockout alleles of *Msh2* were also generated at the Duke site from other lines in the following manner. Mice with an exon of *Msh2* flanked by LoxP sites (obtained from Dr. Raju Kucherlapati under Material Transfer Agreement with Harvard Medical School) were crossed with mice carrying the EIIa-Cre transgene (available from The Jackson Laboratory) to generate the knockout allele of *Msh2*. Mice with a knockout allele of *Trp53* were obtained from the Jackson Laboratory. The genotypes of the animals that underwent the experimental drug treatments were $Pdcd10^{+/-}Trp53^{-/-}$, $Pdcd10^{+/-}Msh2^{-/-}$, $Pdcd10^{+/-}$ and $Krit1^{+/-}Msh2^{-/-}$.

In order to generate these final experimental genotypes, animals of intermediate genotypes were generated in the breeding funnels, as discussed below. These intermediate genotypes were merely used as breeders and did not undergo treatments or procedures. All mutant lines were maintained in the C57BL/6J inbred strain background, also obtained from The Jackson Laboratory. Mice of both sexes were used. Experimental animals were bred and aged up to 5 months before being euthanized. Breeders were kept for up to 8 months before being retired, whereupon they were euthanized. A two-generation breeding scheme is required to produce mice with the final experimental genotypes ($Pdcd10^{+/-}Trp53^{-/-}$, $Pdcd10^{+/-}Msh2^{-/-}$ and $Krit1^{+/-}Msh2^{-/-}$). In the final cross, due to Mendelian segregation of the two mutant alleles, 1 in 8 animals produced the desired genotype. In both the first and second crosses, animals of undesired genotypic combinations were identified before weaning by PCR genotyping, and euthanized.

Groups of mice to be compared were raised and treated contemporaneously. Both simvastatin and atorvastatin, but not fasudil, significantly reduced the mean body weights of the combined $Pdcd10^{+/-}Trp53^{-/-}$ and $Pdcd10^{+/-}Msh2^{-/-}$ mice from 2-4 months of age (Table I). Only simvastatin, but not fasudil and atorvastatin, significantly reduced the mean body weights of $Pdcd10^{+/-}$ mice from 2-5 months of age (Table II). Atorvastatin significantly reduced the mean body weights of $Krit1^{+/-}Msh2^{-/-}$ mice at 2, 3 and 5, but not 4, months of age (Table III). We noted when the mice either died before completing treatment, or were euthanized after completing treatment or suffering from a debilitating illness before completion of treatment (Tables IV and V). The treatment duration per intention to treat (range, mean and median) is summarized in Table VI.

Per animal care guidelines to reduce pain and suffering of the experimental mice, we specifically marked and carefully monitored any mouse that displayed weight loss, labored breathing, hunched posture, lethargy, or a number of other signs of poor health. If the animal's health did not improve or continued to decline, it was sacrificed to reduce its suffering. Since brain tissue from a dead animal deteriorates quite rapidly, such that an animal that dies overnight cannot provide usable tissue by the morning, this policy also enabled us to identify animals that were likely to die just prior to their final endpoint (120 days) and obtain intact brains from them for phenotypic measurements.

As recommended by National Institute of Neurological Disorders and Stroke guidelines, subgroup analyses were planned to glean any treatment effect related to the animal’s sex (Tables

VII through IX). The experiments involving *Pdcd10*$^{+/-}$ mice bred in sensitized backgrounds included 53 *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ (45 males, 8 females) and 6 *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ (5 males, 1 female). *Trp53*$^{-/-}$ females are more susceptible to neural tube defects during fetal development[1] and this results in a skewed sex ratio towards males in the births - that is fewer females born alive than males.[2, 3] Hence it appears that in general *Trp53*$^{-/-}$ females are more fragile to neural tube defects during development and are fewer in number than males in the births, and that in combination with other genes, this sex skewing can be exacerbated, sometimes even to the point of complete loss of any females.[4, 5] The skewed ratios we observed with the *Pdcd10* gene may be due to just the normal fact of skewed ratios from loss of *Trp53* or possibly due to an interaction with *Pdcd10* heterozygosity that is making the skewing even worse. But regardless, the female lethality effect has been seen before with *Trp53* null mice.

Primary and Secondary Outcomes and Statistical Analysis

For primary outcome assessment, we hypothesized that the CCM lesion burden determined by micro-computed tomography at the conclusion of treatment will be significantly decreased in each of 3 respective drug-treated groups as compared to placebo control mice receiving the same drug-free diet and drinking water. Power calculations assumed an effect size of 1.27, determined from similar studies in the *Pdcd10*$^{+/-}$ model comparing CCM lesion burden in placebos with mice depleted of B cells.[6] A low rate of *Pdcd10*$^{+/-}$ mice bred in sensitized backgrounds *Trp53*$^{-/-}$ and *Msh2*$^{-/-}$ were produced since only 1 out of 8 mice had the correct genotype in the final cross and the lack of *Trp53*$^{-/-}$ females born alive for these models, as was indicated previously. Hence, in order to conserve the number of animals in these models, we adjusted the numbers of mice in placebo group to be substantially greater than numbers of mice in the 3 drug-treated groups. Based on an effect size of 1.27, sample size was calculated to be 16 per placebo group and 9 per drug-treated group using the two-sample t test for mean difference to test the significance between the drug-treated and placebo groups (α=0.05, 1-β=0.83, 2-tailed). In the actual study using these models, with 9 fasudil-, 11 simvastatin-, 10 atorvastatin-treated mice compared with 16 placebos, power was at least 83% for α=0.05 assuming the effect size of 1.27. Once animals were included in a study, they were allowed to complete their treatment arm.

Because of the greater rate of mice produced with genotypes *Krit1*$^{+/-}$*Msh2*$^{-/-}$, at least 15 mice were included per group. Based on the same effect size of 1.27, sample size was calculated to be 15 per group using the two-sample t test for mean difference to test the significance between the Rock inhibited (by atorvastatin) and control groups (α=0.05, 1-β=0.92, 2-tailed).

Because of the even greater rate of mice produced with genotype *Pdcd10*$^{+/-}$ than the other 3 genotypes described previously, at least 21 of these mice were included per group. Based on the same effect size of 1.27, sample size was calculated to be 21 per group using the two-sample t test for mean difference to test the significance between the drug-treated and placebo groups (α=0.01, 1-β=0.92, 2-tailed).

Unlike the normally distributed lesion burden data in the B cell depletion studies on *Pdcd10*$^{+/-}$ mice previously reported[6] in which the sample size calculations for the present studies were based upon, the lesion burden data in all of the present studies were not normally distributed. Hence, non-parametric tests were used to test for statistical significance. In situations when most of data per group was non-zero, the 2-sided Conover 2-sample test[7] was used to test for significant differences per group – a test in which sensitivity was improved because the individual data points are weighted. The Conover test is an enhanced version of the Mann-

Whitney U test by weighing the ranks of the original values. It provides an improved use of the non-zero data. If one of the groups contained more zero values than non-zero values, the Mann-Whitney U test was used to test for significant differences per group to avoid possible statistical errors due to over emphasis of weighted outlying data points when analyzed by the 2-sided Conover 2-sample test. Similar non-parametric statistical analyses were conducted with the non-heme iron deposition data, in which these data were not normally distributed as well.

Other prespecified secondary outcome analyses also used appropriate statistical tests. Since the body weights of mice were normally distributed, parametric tests were used to test for statistical significance. The F test was used to evaluate the variances between two unpaired groups. The differences between the two groups were compared using Student's t-test with equal variances and Welch's t test with unequal variances. The log-rank (Mantel-Cox) test was used to compare the survival of animals between treatment groups. The 2-sided Conover 2-sample test was used to compare the proportion of endothelial cells and leukocytes with ROCK activity in CCM lesions per animal between treated and placebo control groups. Analysis of variance was used to compare the durations of treatments.

Statistical analyses were performed using SAS9.4 (SAS Institute Inc., Cary, NC), R v3.4.4 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 7.00 (GraphPad Software Inc., La Jolla, CA). All probability (*P*) values were considered to be statistically significant at $P < 0.05$.

Supplemental Tables

Table I. Body Weights of Combined *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ and *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ Mice

Age		Placebo		Fasudil	*P*-value		Simvastatin		*P*-value		Atorvastatin		*P*-value	
(mo)	n	Mean ± SD	n	Mean ± SD	F	t	n	Mean ± SD	F	t	n	Mean ± SD	F	t
2	8	23.0 ± 4.5	6	22.3 ± 5.1	0.72	0.80	8	17.0 ± 3.9	0.72	0.01	5	15.4 ± 3.5	0.67	< 0.01
3	7	27.8 ± 3.1	3	29.0 ± 4.6	0.39	0.64	5	20.0 ± 5.5	0.21	0.01	3	21.0 ± 5.3	0.26	0.03
4	16	26.8 ± 3.0	9	27.5 ± 4.8	0.13	0.66	11	21.1 ± 4.4	0.19	< 0.001	10	22.5 ± 2.9	0.95	< 0.01

n indicates the number of animals per group: SD, standard deviation
P-values are for the comparison between treatment vs. placebo. The F test was used to evaluate the variances between two unpaired groups. The differences between the two groups were compared using Student's t-test with equal variances and Welch's t test with unequal variances.

Table II. Body Weights of *Pdcd10*$^{+/-}$ Mice

Age	Placebo		Fasudil		*P*-value		Simvastatin		*P*-value		Atorvastatin		*P*-value	
(mo)	n	Mean ± SD	n	Mean ± SD	F	t	n	Mean ± SD	F	t	n	Mean ± SD	F	t
2	13	20.8 ± 2.4	19	21.7 ± 2.7	0.75	0.38	13	20.5 ± 2.3	0.83	0.74	17	18.5 ± 3.1	0.38	0.04
3	16	23.6 ± 3.0	20	24.8 ± 3.1	0.85	0.24	17	24.0 ± 2.5	0.46	0.64	19	20.8 ± 3.4	0.64	0.02
4	19	24.8 ± 3.2	20	26.2 ± 3.6	0.65	0.20	16	26.1 ± 2.6	0.41	0.21	17	21.6 ± 4.2	0.27	0.02
5	21	25.6 ± 3.8	20	26.7 ± 3.6	0.80	0.34	19	26.0 ± 3.1	0.38	0.70	21	22.5 ± 3.7	0.92	0.01

n indicates the number of animals per group: SD, standard deviation
P-values are for the comparison between treatment vs. placebo. The F test was used to evaluate the variances between two unpaired groups. The differences between the two groups were compared using Student's t-test with equal variances and Welch's t test with unequal variances.

Table III. Body Weights of *Krit1*$^{+/-}$*Msh2*$^{-/-}$ Mice

Age		Placebo		Atorvastatin	*P*-value	
(mo)	n	Mean ± SD	n	Mean ± SD	F	t
2	16	20.4 ± 2.6	13	16.4 ± 3.3	0.38	< 0.01
3	16	23.0 ± 2.7	11	20.1 ± 3.9	0.19	0.03
4	15	25.0 ± 3.5	12	23.7 ± 3.7	0.80	0.36
5	19	25.6 ± 3.7	16	22.9 ± 3.8	0.86	0.04

n indicates the number of animals per group: SD, standard deviation; ND, not determined
P-values are for the comparison between atorvastatin treatment vs. placebo. The F test was used to evaluate the variances between two unpaired groups. The differences between the two groups were compared using Student's t-test with equal variances and Welch's t test with unequal variances.

Table IV: Number of Combined *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ and *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ Mice Not Surviving the Complete Treatment with the Indicated Features of Attrition*

Number of mice that started placebo treatment	20
Total attrition†	4
Brain hemorrhage	1
Systemic illness/tumor	1
No information/other	2
Number of mice that started fasudil treatment	9
Total attrition	0
Brain hemorrhage	0
Systemic illness/tumor	0
No information/other	0
Number of mice that started simvastatin treatment	13
Total attrition	2
Brain hemorrhage	1
Systemic illness/tumor	1
No information/other	0
Number of mice that started atorvastatin treatment	17
Total attrition‡	7
Brain hemorrhage§	2
Systemic illness/tumor‖	1
No information/other	4

*The Fisher exact test was used to analyze the attrition differences between atorvastatin vs. placebo, combined atorvastatin and simvastatin vs. placebo, and combined atorvastatin and simvastatin vs. combined placebo and fasudil treatment groups. In all comparisons, $P>0.21$ for numbers of mice that underwent attrition vs. those numbers surviving and $P>0.58$ for numbers of mice with brain hemorrhage vs. those numbers without brain hemorrhage.
†Includes 4 *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ mice
‡Includes 5 *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ and 2 *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ mice
§Includes 2 *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ mice
‖Includes 1 *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ mouse

Table V: Number of *Krit1*$^{+/-}$*Msh2*$^{-/-}$ Mice Not Surviving the Complete Treatment with the Indicated Features of Attrition

Number of mice that started the placebo treatment	26
Total attrition	7
Brain hemorrhage	2
Systemic illness/tumor	3
No information/other	2
Number of mice that started atorvastatin	29
Total attrition	13
Brain hemorrhage	3
Systemic illness/tumor	4
No information/other	6

Table VI: Range, Mean and Median for Duration of Treatment for Placebo, Fasudil, Simvastatin and Atorvastatin in Combined *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ and *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$, *Krit1*$^{+/-}$*Msh2*$^{-/-}$ and non-Sensitized *Pdcd10*$^{+/-}$Models*

Mouse strain	Treatment	Range (days)	Mean (days)	Median (days)
Pdcd10$^{+/-}$*Trp53*$^{-/-}$/*Pdcd10*$^{+/-}$*Msh2*$^{-/-}$				
	Placebo	90-121	95.5	91.5
	Fasudil	91-112	95.1	94.0
	Simvastatin	90-103	94.4	94.0
	Atorvastatin	83-126	98.4	91.5
Krit1$^{+/-}$*Msh2*$^{-/-}$				
	Placebo	121-134	123.1	122.0
	Atorvastatin	113-129	122.8	122.5
Pdcd10$^{+/-}$				
	Placebo	119-123	120.3	120.0
	Fasudil	120-127	121.5	120.0
	Simvastatin	120-142	123.8	121.0
	Atorvastatin	120-143	123.3	121.0

*No significant differences were observed by analysis of variance

Table VII. Lesion Burden and Non-Heme Iron per Animal in Both Sexes of Combined *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ and *Pdcd10*$^{+/-}$*Msh2*$^{-/-}$ Mice

Sex	Placebo		Fasudil			Simvastatin			Atorvastatin		
	n	Mean ± SD	n	Mean ± SD	*P*-value	n	Mean ± SD	*P* – value	n	Mean ± SD	*P*-value
Lesion Volume/Brain Volume per Animal											
Male	15	0.00957 ± 0.01772	8	0.00467 ± 0.00567	0.039	6	0.00625 ± 0.00930	0.302	10	0.00470 ± 0.00593	0.027
Female	1	0.00140	1	0.00023	ND	5	0.01728 ± 0.03728	0.246	0	ND	ND
Non-Heme Iron per Animal											
Male	15	200637 ± 4417523	8	83333 ± 97468	0.028	6	98607 ± 134051	0.106	10	55709 ± 81263	0.007
Female	1	665	1	1152	ND	5	36845 ± 82064	0.237	0	ND	ND

n indicates the number of animals per group: SD, standard deviation; ND, not determined
P-values are for the comparison between treatment vs. placebo. The 2-sided Conover 2-sample test was used to assess for significant differences.

Table VIII. Lesion Burden and Non-Heme Iron per Animal in Both Sexes of *Krit1*$^{+/-}$*Msh2*$^{-/-}$ Mice

		Placebo		Atorvastatin	
Sex	n	Mean ± SD	n	Mean ± SD	*P*-value
Lesion Volume/Brain Volume per Animal (X 10^6)					
Male	11	62.00 ± 95.02	9	2.70 ± 4.14	0.016
Female	8	40.24 ± 95.44	7	2.50 ± 4.06	0.392
Non-Heme Iron per Animal					
Male	11	969 ± 1871	9	0 ± 0	0.218
Female	8	161664 ± 455296	7	0 ± 0	0.467

n indicates the number of animals per group: SD, standard deviation
P-values are for the comparison between atorvastatin treatment vs. placebo. The 2-sided Conover 2-sample test was used to assess for significant differences.

Table IX. Lesion Burden and Non-Heme Iron per Animal in Both Sexes of *Pdcd10* Mice

Sex	Placebo		Fasudil			Simvastatin			Atorvastatin		
	n	Mean ± SD	n	Mean ± SD	*P* - value	n	Mean ± SD	*P*- value	n	Mean ± SD	*P*- value
Lesion Burden per Animal ($X10^3$)											
Male	10	0.0039 ± 0.0118	15	0.0153 ± 0.0332	0.638	15	0.0916 ± 0.3252	0.487	8	0. 0160 ± 0.0357	0.084
Female	11	0.0520 ± 0.0793	7	0.0925 ± 0.1745	0.584	6	0.0040 ± 0.0051	0.175	13	0.1329 ± 0.2918	0773
Non-Heme Iron per Animal											
Male	10	14 ± 43	15	782 ± 2293	0.447	15	4017 ± 14871	0.357	8	1856 ± 2366	0.018
Female	11	785 ± 1204	7	209 ± 276	0.396	6	733 ± 1196	0.551	13	1715 ± 5596	0.116

n indicates the number of animals per group: SD, standard deviation
P-values are for the comparison between treatment vs. placebo. The 2-sided Conover 2-sample test was used to assess for significant differences.

Supplemental Figures and Figure Legends

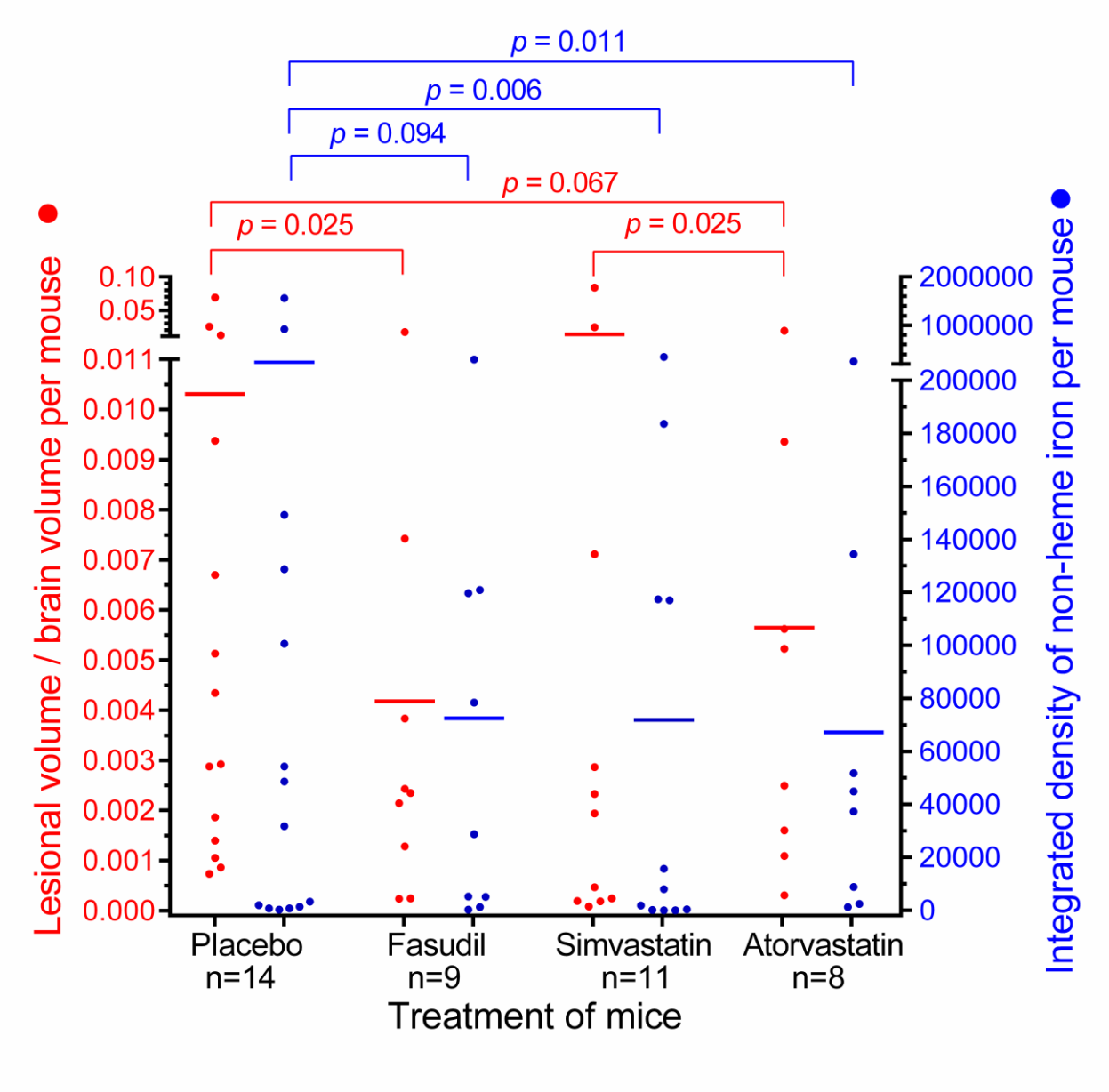

Figure I. Rock inhibition diminishes lesion burden (left axis, red) and lesion hemorrhage (right axis, blue) in the *Pdcd10*$^{+/-}$*Trp53*$^{-/-}$ model. Treatment with fasudil, but not simvastatin, decreased lesion burden compared to contemporaneously raised placebos. There was a trend for decreased lesion burden with atorvastatin treatment compared to contemporaneously raised placebos. Compared with placebos, treatment of mice with any of 3 Rock inhibitors decreased non-heme iron deposition in lesions. The 2-sided Conover 2-sample test was used to assess for significance.

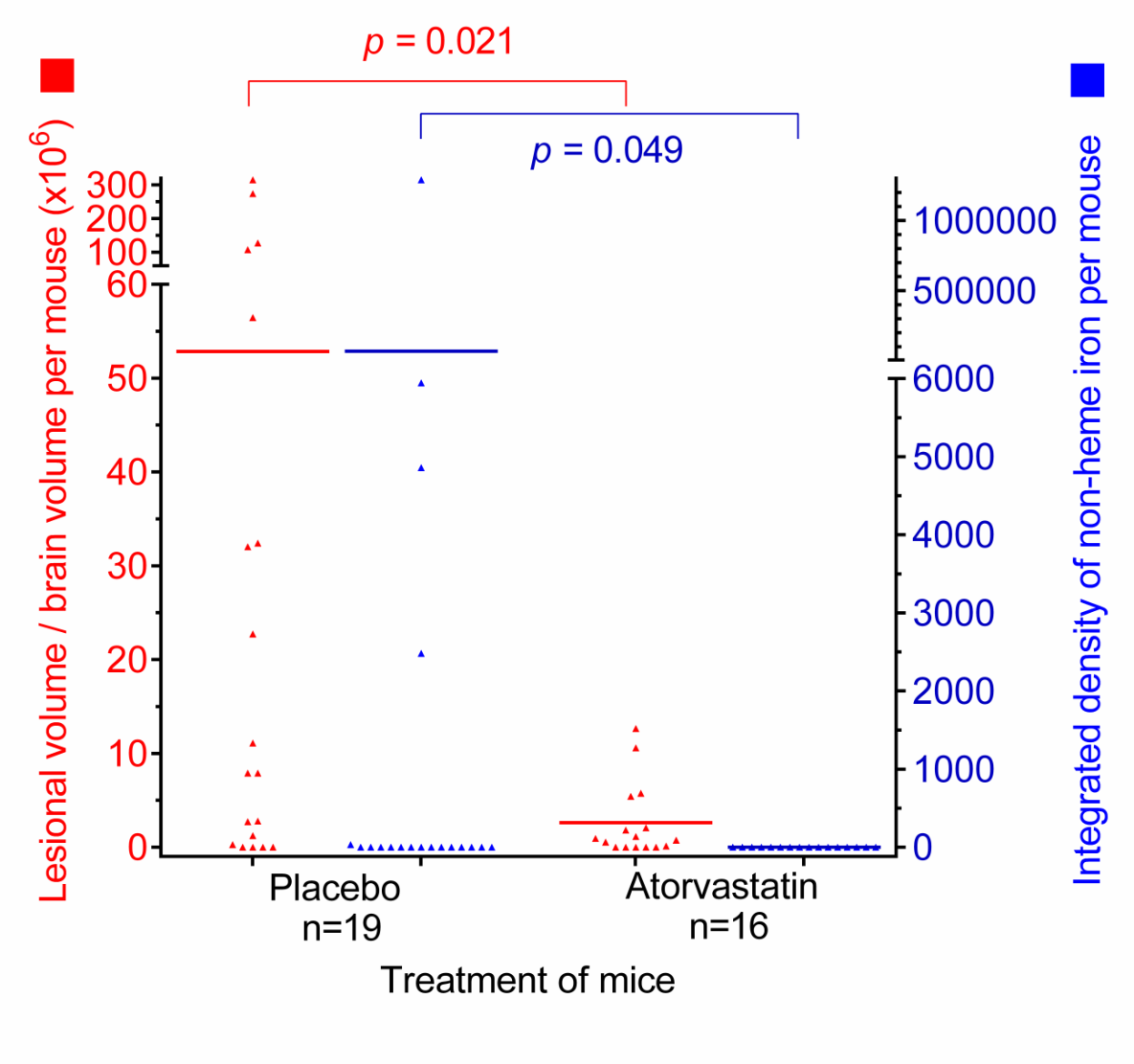

Figure II. Lesion burden and lesion hemorrhage in *Krit1*$^{+/-}$*Msh2*$^{-/-}$ models. Treatment with atorvastatin decreased lesion burden by 20-fold (left axis, red) and completely abrogated non-heme iron (right axis, blue) compared with placebos. The Mann-Whitney U test was used to assess for significant differences.

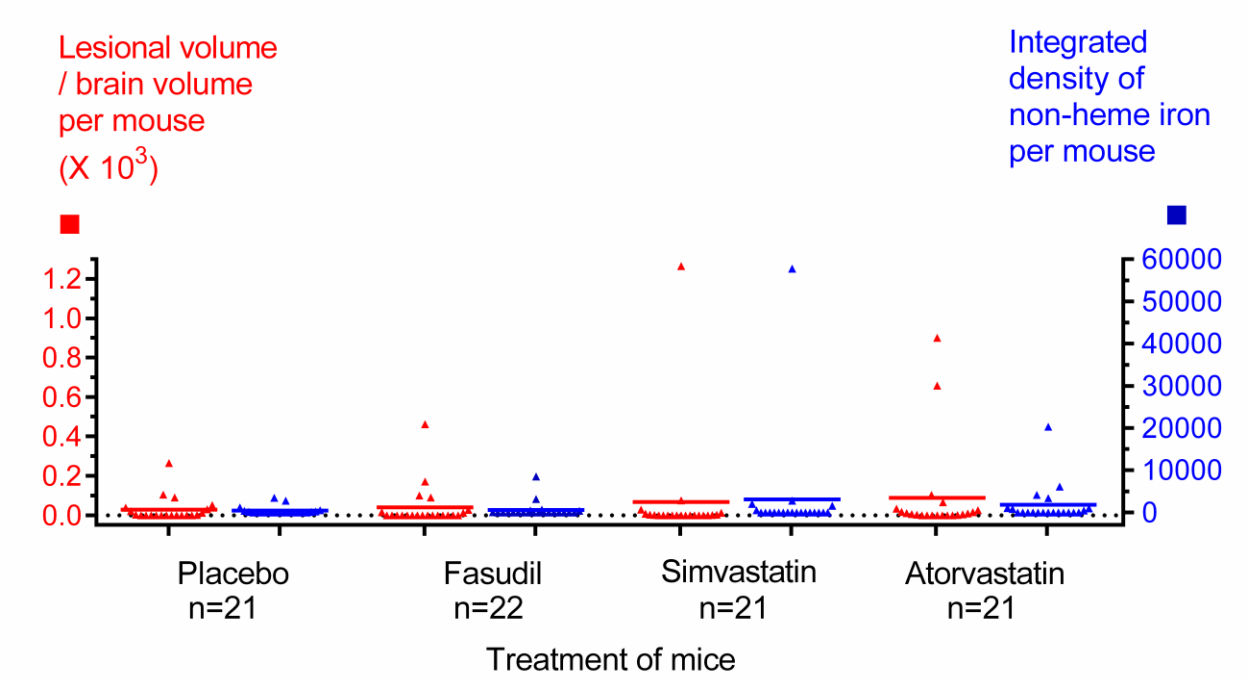

Figure III. Lesion burden and lesion hemorrhage in *Pdcd10*$^{+/-}$ models. Treatment with the Rock inhibitors, fasudil, simvastatin or atorvastatin did not affect lesion burden (left axis, red) or non-heme iron (right axis, blue) compared with placebos. The Mann-Whitney U test was used to assess for significant differences.

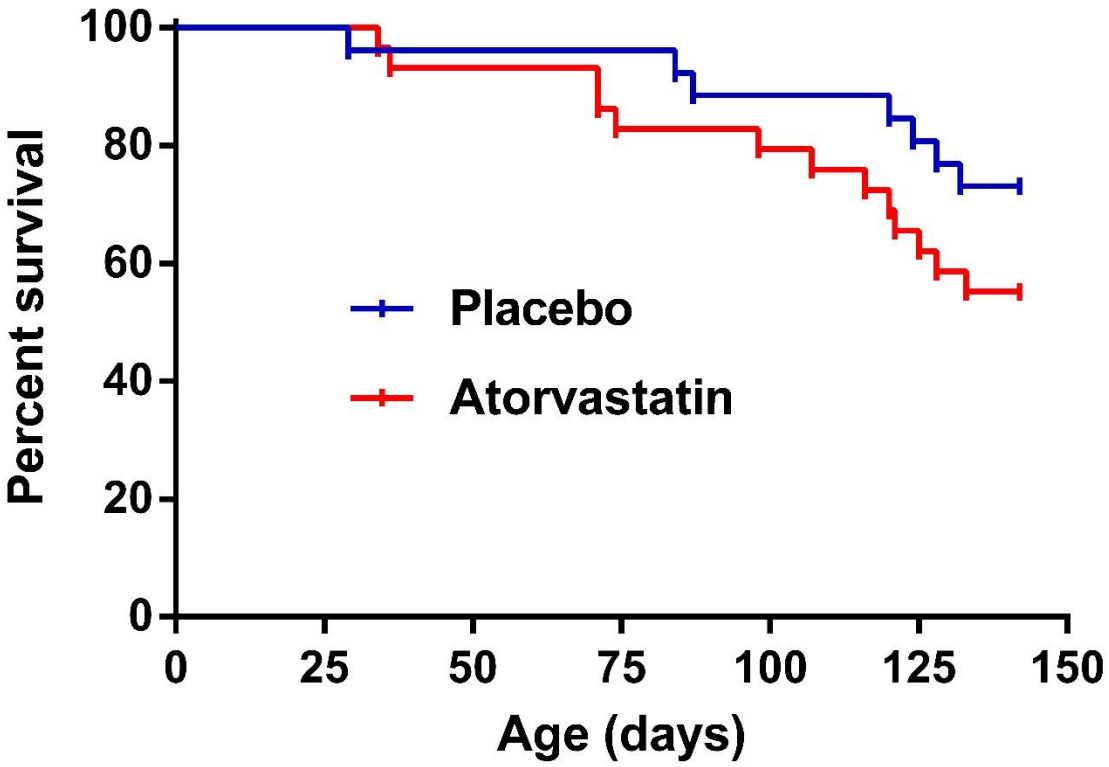

Figure IV. Attrition in *Krit1*$^{+/-}$*Msh2*$^{-/-}$ models. Kaplan-Meier plots show no significant effect (*P*=0.156) of atorvastatin treatment (n=29) on survival compared with placebos (n=26) from weaning to the earliest age for the end of treatment (142 days of age). The log-rank (Mantel-Cox) test was used to assess for significant differences.

Supplemental References

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*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	Yes

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	Yes

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	Yes
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	Yes
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	Yes

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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