ONLINE SUPPLEMENT

ROCK Inhibition with Fasudil versus Simvastatin in Murine Models of Cerebral Cavernous Malformations

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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 knockout alleles of *Ccm1* and *Ccm2* were generated 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 $Ccm1^{+/-}Msh2^{-/-}$ and $Ccm2^{+/-}Trp53^{-/-}$.

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 to up to 6 months before being sacrificed. Breeders were kept for up to 8 months before being retired, whereupon they were sacrificed. A two-generation breeding scheme is required to produce mice with the final experimental genotypes ($Ccm1^{+/-}Msh2^{-/-}$ and $Ccm2^{+/-}Trp53^{-/-}$). 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.

Figure I presents the schema of concurrent random assignment of animals to placebo or treatment groups.

Primary and Secondary Outcomes and Statistical Analysis

Groups of mice to be compared were raised and treated contemporaneously. Fasudil or simvastatin treatment did not affect the body weight of the animals when compared to placebos in any of the experimental groups (Table I). 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 (Table II). As recommended by NINDS guidelines, subgroup analyses were planned to glean any treatment effect related to the animal's sex (Tables III and IV).

For primary outcome assessment, we hypothesized that the prevalence of mature stage 2 CCM lesions at the conclusion of treatment will be decreased by 50% in each respective drug-treated group as compared to control mice receiving the same drug-free diet and drinking water. Power calculations assumed that 50% of mice will harbor one or more mature stage 2 CCM lesions (defined below) after age 4 months in the placebo group, with a median of 3 lesions per brain, which are conservative estimates based on our preliminary studies.[1] Based on data from preliminary studies with a much smaller number of animals,[1] initial sample size was calculated to be 20 per group using Poisson maximum likelihood to test the significance between the drug-treated and placebo groups (α=0.05, 1-β=0.88, 2-tailed). We hence commenced randomized assignment to treatment groups in the first experiment with a larger group of mice. This accounts for the larger samples sizes in the $Ccm1^{+/-}Msh2^{-/-}$ Fasudil and simvastatin comparisons. Once

animals were included in a study, they were allowed to complete their treatment arm. Based on results of the *Ccm1*$^{+/-}$*Msh2*$^{-/-}$ Fasudil experiments, we calculated new sample size estimates to be 12 per group within even greater significance between the drug-treated and placebo groups (α=0.01, 1-β=0.94, 2-tailed). We hence used the smaller sample size for the later *Ccm2*$^{+/-}$*Trp53*$^{-/-}$ simvastatin comparison to placebo.

Since the data had fit the Poisson model well in our preliminary studies,[1] we used the Poisson maximum likelihood test to assess for statistical differences in lesion number per brain between drug-treated and placebo mice in early- and late-treated groups. For assessment of primary outcome, the number of stage 2 lesions per animal between ROCK inhibited and placebo groups were compared using Negative Binomial Regression if the outcome was over-dispersed and Poisson regression analysis if the mean and variance were equal.

For other prespecified secondary outcome analyses, lesional areas were compared among the different treatment groups. 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 Mann-Whitney test was used to compare integrated density of iron per lesion and integrated density of iron per lesional area between treatment groups. The chi-square test was conducted to compare the prevalence of stage 2 lesions among all lesions, and the proportion of endothelial cells with ROCK activity in stage 2 lesions between treatment groups. And the log-rank (Mantel-Cox) test was used to compare the survival of animals between treatment groups.

The DerSimonian and Laird method[2] was used for the meta-analyses for lesion burden, lesional area and iron deposition incorporating effect in the two genotypes. The weights of the *Ccm1* or *Ccm2* groups were calculated with the inverse of respective variance (including within each individual study variance and between studies variance). Heterogeneity was assessed by the chi-square test and quantified by the inconsistency index I^2, which is the percentage of variance across the *Ccm1* and *Ccm2* groups.

Statistical analyses were performed using SAS9.4 (SAS Institute Inc., Cary, NC), R v3.2.3 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA). All probability (*P*) values were considered to be statistically significant at $P< 0.05$.

Supplemental Tables

Table I. Weights of Murine Models Receiving Placebo vs. Fasudil or Simvastatin

Genotype	Treatment (duration)	Age at weighing	Mice given placebo			Treated mice			Probability
			n	weight*	SD	n	weight*	SD	
Ccm1$^{+/-}$*Msh2*$^{-/-}$	Fasudil (weaning - 5 mo)	2 mo	7	22.9	3.0	13	22.6	3.5	0.88
		3 mo	8	25.6	5.7	12	24.8	4.2	0.70
		4 mo	12	27.1	6.1	12	27.1	4.5	1.00
		5 mo	12	25.7	6.0	16	27.5	6.5	0.46
Ccm1$^{+/-}$*Msh2*$^{-/-}$	simvastatin (weaning - 5 mo)	2 mo	11	19.7	2.2	27	18.6	4.1	0.39
		3 mo	14	22.2	2.4	27	21.1	3.3	0.28
		4 mo	16	25.2	5.3	28	23.4	3.5	0.20
		5 mo	15	23.6	4.7	28	23.5	3.5	0.93
Ccm1$^{+/-}$*Msh2*$^{-/-}$	Fasudil (3 mo - 4 mo)	2 mo	9	19.9	2.0	8	20.0	2.9	0.93
		3 mo	9	22.8	2.5	10	22.5	3.1	0.83
		4 mo	13	25.5	5.8	15	23.6	3.8	0.32
Ccm2$^{+/-}$*Trp53*$^{-/-}$	Fasudil (weaning - 5 mo)	2 mo	17	25.3	4.3	19	26.6	2.4	0.25
		3 mo	11	27.4	6.7	16	30.1	1.9	0.13
		4 mo	15	28.8	6.5	15	31.7	5.1	0.17
Ccm2$^{+/-}$*Trp53*$^{-/-}$	simvastatin (weaning - 5mo)	2 mo	11	22.1	2.5	12	21.4	3.3	0.58
		3 mo	11	24.6	3.6	12	24.9	3.5	0.85
		4 mo	11	25.5	3.2	12	24.2	3.7	0.39

n indicates the number of animals; SD, standard deviation; mo, months
*mean weight in grams

Table II: Number of Mice Not Surviving the Complete Treatment with the Indicated Features of Attrition

Mouse strain	*Ccm1*$^{+/-}$*Msh2*$^{-/-}$			*Ccm2*$^{+/-}$*Trp53*$^{-/-}$	
Treatment groups	Placebo vs. Fasudil	Placebo vs. simvastatin	Placebo vs. Fasudil	Placebo vs. Fasudil	Placebo vs. simvastatin
Duration of treatment	Weaning to 4-5 months	Weaning to 4-5 months	3 months to 4 months	Weaning to 4-5 months	Weaning to 4-5 months
Total number of mice that started the placebo treatment	25	27	20	24	12
Total attrition (placebo)	9	7	4	6	1
Brain hemorrhage	3	4	4	2	0
Systemic illness/tumor	1	0	0	1	0
No information/other	5*	3†	0	3§	1
Total number of mice that started the drug treatment	25	42	18	24	12
Total attrition (drug-treated)	3	14	1	6	0
Brain hemorrhage	2	4†	0	4	0
Systemic illness/tumor	0	3	1	2	0
No information/other	1	7‡	0	0	0

*One/‡three mice with overgrowth of teeth (malocclusion).
†One mouse with hydrocephalus.
§One mouse with terminal brain pathology, including dilated cerebral vessels, possible CCM

Table III. Number of Lesions Per Animal in Both Sexes

			Fasudil					Simvastatin				
CCM Stage	Genotype	Sex	Placebo		Treated		p-value	Placebo		Treated		p-value
			n	mean ± SEM	n	mean ± SEM		n	mean ± SEM	n	mean ± SEM	
Stage 2	*Ccm1*$^{+/-}$*Msh2*$^{-/-}$	Male	9	0.56 ± 0.24	9	0.11 ± 0.11	0.14	10	0.20 ± 0.20	14	0.57 ± 0.25	0.36
		Female	7	0.86 ± 0.34	13	0.23 ± 0.12	0.06	10	0.00 ± 0.00	14	0.21 ± 0.11	0.48
	Ccm2$^{+/-}$*Trp53*$^{-/-}$	Male	14	0.93 ± 0.44	18	0.33 ± 0.18	*0.04	9	0.22 ± 0.15	9	0.44 ± 0.44	0.42
		Female	4	0.25 ± 0.25	0	ND	ND	2	2.50 ± 2.50	3	0.00 ± 0.00	†0.46
All lesions (Stage 1&2)												
	Ccm1$^{+/-}$*Msh2*$^{-/-}$	Male	9	0.67 ± 0.33	9	0.22 ± 0.15	0.18	10	1.60 ± 0.81	14	1.50 ± 0.39	0.91
		Female	7	1.29 ± 1.38	13	1.15 ± 1.34	0.8	10	1.67 ± 0.67	14	1.00 ± 0.23	0.37
	Ccm2$^{+/-}$*Trp53*$^{-/-}$	Male	14	1.43 ± 0.40	18	1.61 ± 0.41	0.68	9	1.22 ± 0.36	9	1.22 ± 0.52	1
		Female	4	0.50 ± 0.29	0	ND	ND	2	4.00 ± 3.00	3	0.67 ± 0.33	*0.02

n indicates the number of animals; SEM, standard error of the mean; ND, not determined, since no females were included in the Fasudil group

*p<0.05

†from Wilcoxon rank sum test, since a p value cannot be calculated from a Poisson distribution assumption

Table IV. Integrated Density of Iron Per Stage 2 Lesional Area in Both Sexes

Genotype	Sex	Fasudil					Simvastatin				
		Placebo		Treated		p-value	Placebo		Treated		p-value
		n	mean ± SEM	n	mean ± SEM		n	mean ± SEM	n	mean ± SEM	
Ccm1$^{+/-}$*Msh2*$^{-/-}$	Male	5	12.7 ± 6.6	1	7.5 ± 0.0	†0.76	3	83.1 ± 43.3	5	8.6 ± 8.6	0.07
	Female	6	172.8 ± 109.5	3	0.005 ± 0.003	*0.02	3	116.9 ± 106.8	2	1.5 ± 1.5	0.4
Ccm2$^{+/-}$*Trp53*$^{-/-}$	Male	13	95.2 ± 40.2	6	79.5 ± 37.8	0.61	2	23.7 ± 10.0	4	6.5 ± 4.1	0.27
	Female	1	161.9 ± 0.0	0	ND	ND	5	130.5 ± 64.0	0	ND	ND

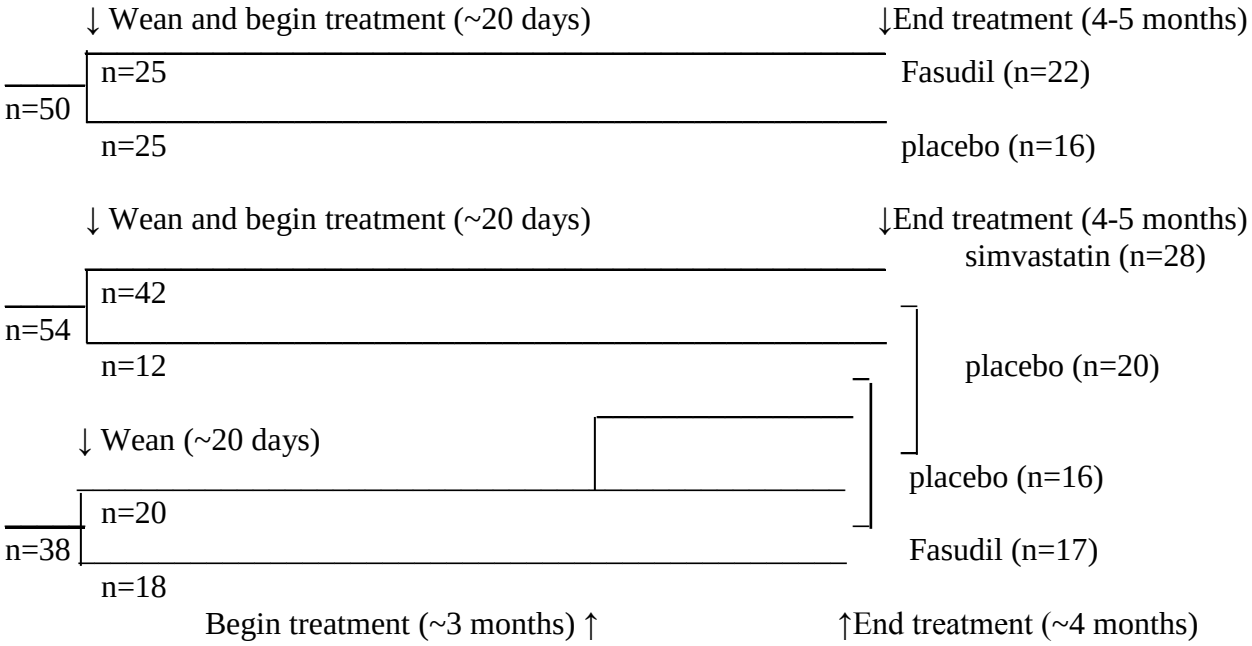
n indicates the number of animals; SEM, standard error of the mean; ND, not determined, since no stage 2 lesions were found in female *Ccm2*$^{+/-}$*Trp53*$^{-/-}$mice for both treatment groups

*p<0.05

†from the general linear model, since a p value cannot be calculated from the Wilcoxon rank sum test

Supplemental Figures and Figure Legends

Treatment Groups in *Ccm1*$^{+/-}$*Msh2*$^{-/-}$ models
(long term Fasudil, long term Simvastatin and late short term Fasudil)



Treatment Groups in *Ccm2*$^{+/-}$*Trp53*$^{-/-}$ models
(long term Fasudil and long term Simvastatin)

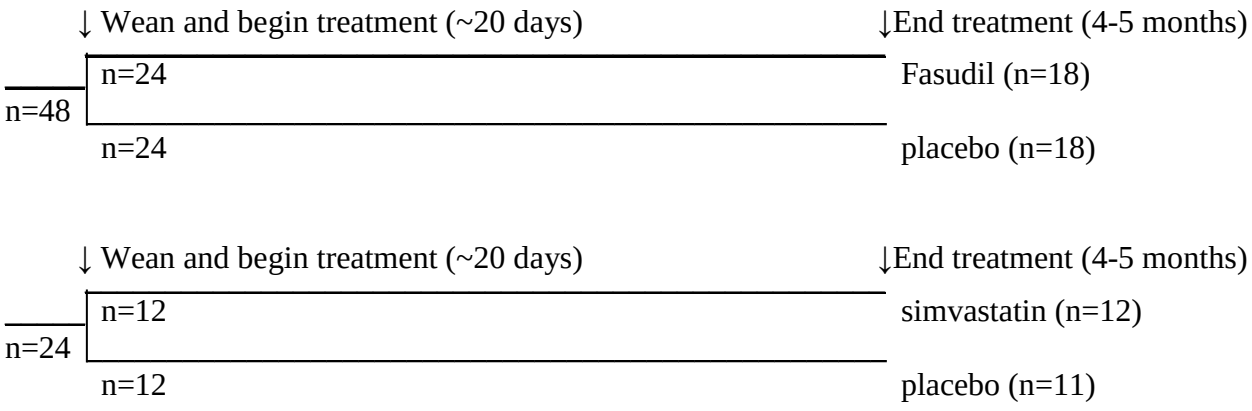


Figure I. Schema summarizing concurrent random assignments of mice with the two genotypes to placebo, Fasudil and simvastatin treatment groups. For the *Ccm1*$^{+/-}$*Msh2*$^{-/-}$ long term simvastatin experiment, contemporaneously raised placebos from the *Ccm1*$^{+/-}$*Msh2*$^{-/-}$ late, short term Fasudil group were added to balance the treatment arms.

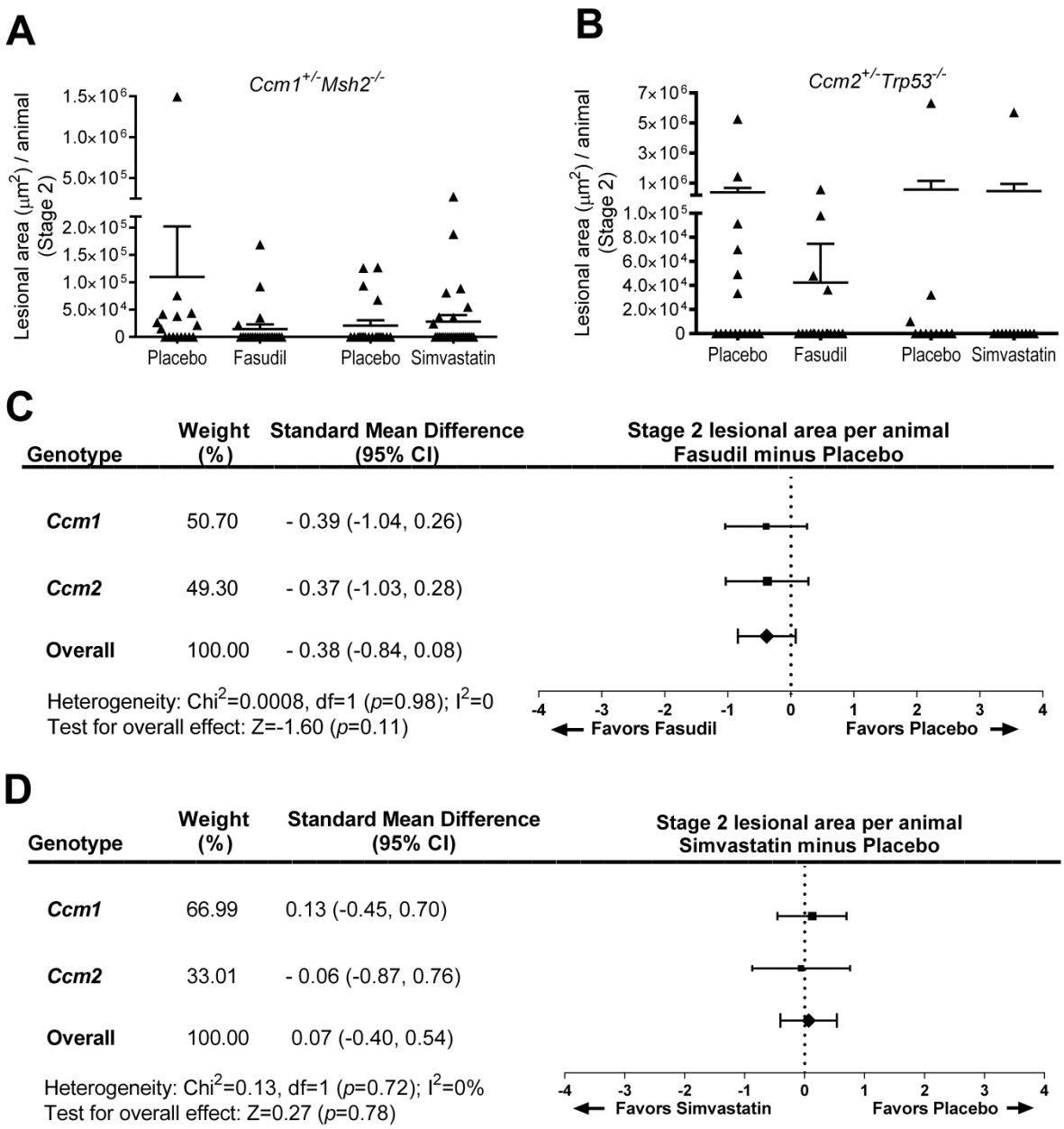


Figure II. Stage 2 lesion area per animal in *Ccm* models with Fasudil and simvastatin treatment. The stage 2 lesion area in *Ccm1*$^{+/-}$*Msh2*$^{-/-}$ models was decreased non-significantly in Fasudil-treated mice (n=22) compared to placebos (n=16), but not affected in simvastatin-treated mice (n=28) compared to placebos (n=20) (**A**) Similarly, the stage 2 area in *Ccm2*$^{+/-}$*Trp53*$^{-/-}$ models was decreased non-significantly in Fasudil-treated mice (n=18) compared to placebos (n=18) but not affected in simvastatin-treated mice (n=12) compared to placebos (n=11). Horizontal bars are means (longer lines) and standard error of the mean (shorter lines). Meta analyses of combined effect in the two genotypes show a non-significant trend for reduced stage 2 lesional area per animal treated with Fasudil (**C**), but not with simvastatin (**D**) compared to placebo.

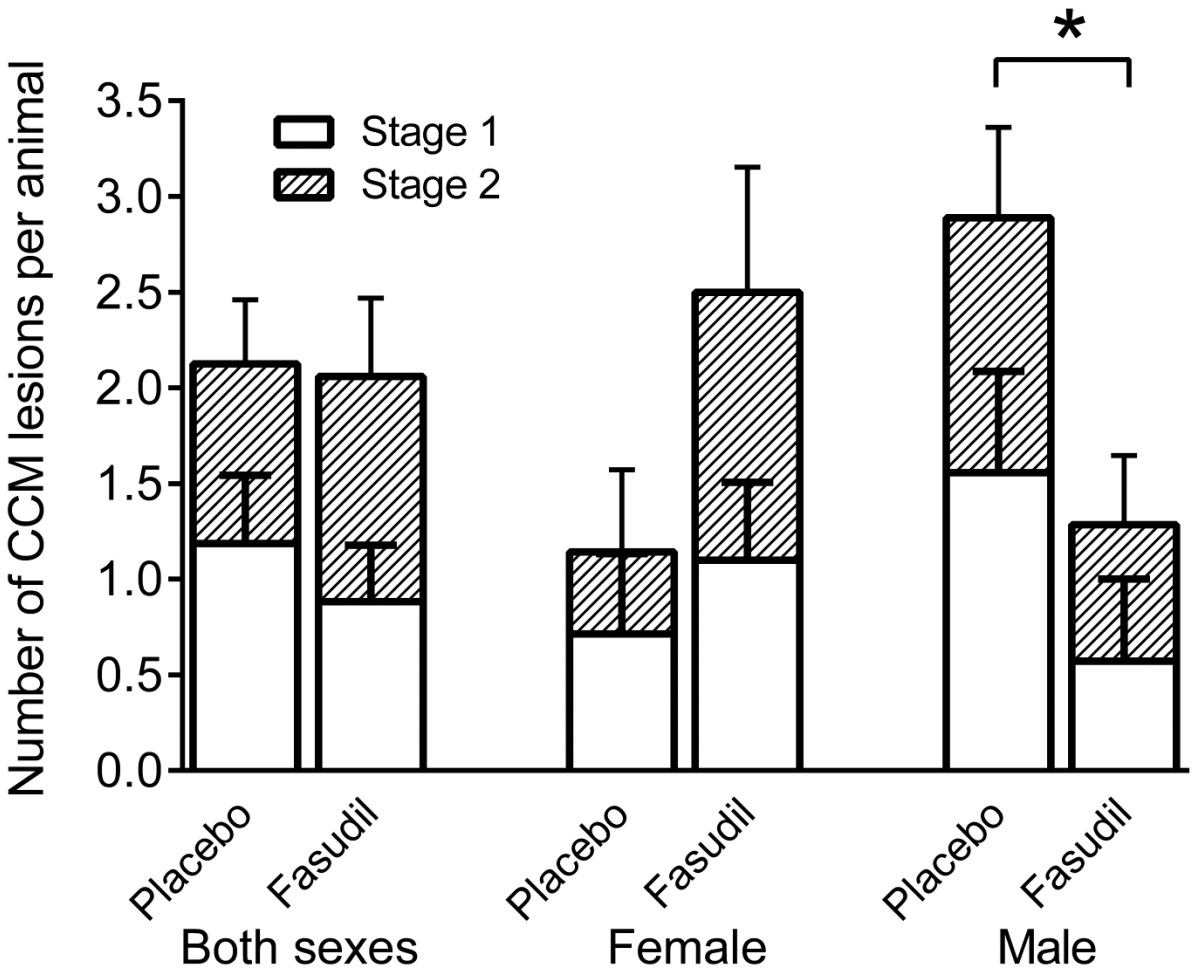
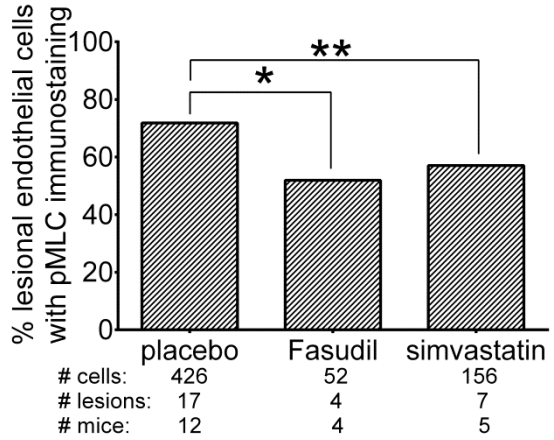
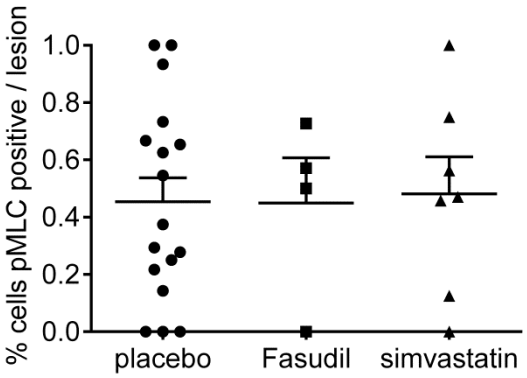


Figure III. Lesion burden in after short-term, late Fasudil treatment of *Ccm1*$^{+/-}$*Msh2*$^{-/-}$ murine models. The number of stage 2 and total lesions were not affected by 1 month of Fausdil (n=17) treatment in mice between 3 and 4 months of age, when compared to placebos (n=16). Fasudil (n=7) significantly decreased the combined stage 1 and stage 2 CCM lesions in males when compared to placebos (n=9), with an opposite trend in females. A male-female imbalance of lesion burden in placebo mice is noted.

A.



B.



C.

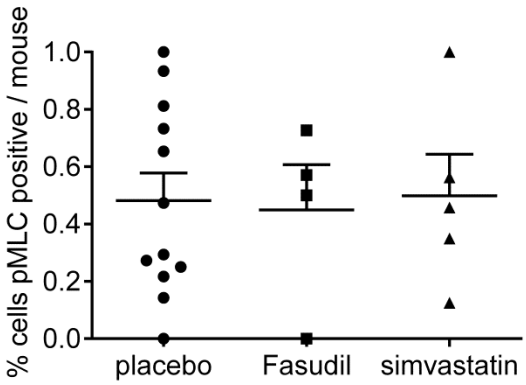


Figure IV. RhoA kinase (ROCK) activity in mature stage 2 lesions with Fasudil and simvastatin treatment. (**A**) The percentage of all lesional endothelial cells with ROCK activity measured by pMLC staining in multicavernous stage 2 lesions in *Ccm1*$^{+/-}$*Msh2*$^{-/-}$ murine models is decreased by Fasudil treatment (**P*=0.003) and by simvastatin treatment (***P*=0.0007). The total number of endothelial cells counted, as well as the number of stage 2 lesions and the number of mice used for the cell counts, are indicated. Comparison of the prevalence of immunopositive cells when analyzed per lesion (**B**) and per mouse (**C**) are not significantly different.

Supplemental References

1. McDonald DA, Shi C, Shenkar R, Stockton RA, Liu F, Ginsberg MH, et al. Fasudil decreases lesion burden in a murine model of cerebral cavernous malformation disease. *Stroke*. 2012;43:571-574.
2. DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials*. 1986;7:177-188.