Analysis of PRT060318 activity on platelet function (whole blood)

Determination of PRT060318 IC_{50} on arterial thrombosis (endpoint measurement)
Human blood was collected in syringes containing 5 μM final concentration of the factor Xa inhibitor C921-78 to preserve physiological Ca^{2+} concentration.[1] Blood was perfused through a collagen-coated capillary at $1500s^{-1}$ for 5 min and end point evaluation of the thrombotic deposits performed as described.[1]

Real-time assessment of PRT060318 effects on the kinetics of thrombosis
Blood was collected in syringes containing 5 μM (human, pigs) or 10 μM (rabbits) of C921-78 and perfused through a collagen-coated capillary at $1500s^{-1}$ (human, pigs) or $1000s^{-1}$ (rabbits, to accommodate lower volume of blood) for a period of 5 min. Platelets were labeled *in situ* with Rhodamine 6G (final concentration 1.25 μg/mL). Deposition of labeled platelets on collagen was visualized using a microscope with a high-power light emitting diode (Luxeon V, Lumileds Lighting, San Jose, CA) coupled to a Sony XCD X-710 digital camera. Images were recorded at a frequency of 1 Hz and thrombus size plotted as fluorescence intensity divided by total area over time. For dethrombosis experiments, collagen-coated chambers were first exposed to untreated samples of blood at arterial shear rates for $250s^{-1}$ then perfused with treated blood (vehicle control or 5 μM PRT060318) at the same shear rate.

Mouse photochemical carotid artery thrombosis
Male C57Bl/6J mice (age 7–9 weeks) were anesthetized by intraperitoneal injection with sodium pentobarbital (62.5mg/kg) and placed in the supine position on a dissecting microscope (Nikon SMZ-2T, Mager Scientific, Inc., Dexter, MI). A midline surgical incision was made to expose the right common carotid artery and a Doppler flow probe (MC 0.5PSL Nanoprobe, Model 0.5 VB, Transonic Systems, Ithaca, NY) was placed under the vessel. The probe was connected to a flowmeter (Transonic Systems Model TS420) and was interpreted with a computerized data acquisition program (Windaq, DATAQ Instruments, Arkron, OH). Rose Bengal at a concentration of 10 mg/mL in phosphate-buffered saline was then injected into the tail vein to administer a dose of 50 mg/kg.[2] The mid portion of the common carotid artery was then illuminated with a 1.5-mW green light laser source (540 nm; Melles Griot**,** Carlsbad, CA) 5 cm from the artery. Blood flow was monitored continuously from the onset of injury. The time to occlusion, determined only after the vessel remained closed with a cessation of blood flow for 10 min, was recorded. PRT060318 (30 mg/kg in distilled water) or vehicle control, were administered via oral gavage 2 hrs prior to carotid injury.

Mouse femoral artery wire injury
Male C57BL/6 mice aged 8 to 10 weeks were anesthetized on day 0 using ketamine (80 mg/kg i.p.) and xylazine (5 mg/kg i.p.), and wire injury of the femoral artery was performed, as described previously.[3, 4] PRT060318 (30 mg/kg in distilled water) or vehicle control, were administered via oral gavage 2 hrs prior to injury and then twice daily for 10d. All animals survived until the time of planned sacrifice without bleeding or infection. Mice were maintained in animal facilities at Case Western Reserve University School of Medicine. Animal care and procedures were reviewed and approved by the Institutional Animal Care and Use Committees and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Femoral artery harvesting and analysis
One day (vehicle: n=6; PRT060318: n=6), 5d (vehicle: n=10; PRT060318: n=10), or 28d (vehicle: n=13, PRT060318: n=15) after vascular injury, anesthesia was administered, the chest cavity opened, and the animals sacrificed by right atrial exsanguination. A 22-gauge butterfly catheter was inserted into the left ventricle for *in situ* pressure perfusion at 100 mm Hg with 0.9% saline for 1 min followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, for 10 min. The right and left femoral arteries were excised and immersed in buffered paraformaldehyde. Spleen and small intestine from three animals were harvested as control tissues for immunohistochemistry. Animals harvested at 5d received BrdU, 50 mg/kg i.p., 18 and 1 h before sacrifice.

Femoral arteries were embedded, and two cross sections cut 1 mm apart were stained with hematoxylin and eosin, and Verhoeff tissue elastin stain. A histologist blinded to treatment group measured the luminal, intimal, and medial areas of each cross-sectional plane using a microscope equipped with a CCD camera (Zeiss AxioCam MRc5) interfaced to a computer running NIH Image. Computer-assisted imaging analysis (Zeiss Axiovision software Rel 4.5) was done by a histologist blinded to genotype. Results for the two planes of each artery were averaged. For immunohistochemistry, standard avidin-biotin procedures for mouse CD45 (leukocyte common antigen, BD Biosciences, San Diego, CA), mouse neutrophils (mAb 7/4, AbD Serotec, North Carolina), mouse macrophages (Mac-3, mAb M3/84, BD Biosciences), mouse platelets (anti-glycoprotein IIb, BD Biosciences), BrdU (DAKO, Carpinteria, CA), and phospho-Vav (Santa Cruz) were used. For each antibody, controls included species specific non-immune IgG as well as omission of the primary antibody. For CD45, mAb 7/4, and BrdU, immunostained sections were quantified as the number of immunostained positive cells/total number of nuclei. For Mac-3, phospho-Vav, and GPIIb, staining was quantified as the percent positive staining area as the fraction of immunopositive staining to total area measured.

Atherosclerosis experiments: ApoE model of high-fat diet-induced atherosclerosis
To induce atherosclerosis, 8-week-old male $ApoE^{-/-}$ mice consumed a high-fat diet (Clinton/Cybulsky Rodent Diet D12108 with 1.25% cholesterol, Research Diets New Brunswick, NJ) for 20 weeks, as described previously.[5] PRT060318 (30 mg/kg in distilled water, n=16) or vehicle control (n=16), were administered via oral gavage twice daily for 3 weeks followed by 1 week off for a total of 16 weeks. At the time of harvest, mice were euthanized by CO_2 asphyxiation prior to *in situ* perfusion fixation as described above.

The aortas were cleaned of excess adventitial tissue, and then stained in 0.5% Sudan IV (Sigma) for 15 minutes. After staining, aortas were differentiated in 80% ethanol prior to placement in buffered solution. Aortas were analyzed after they were opened longitudinally and pinned. Images of Sudan IV-stained aortas were taken with a digital camera (Nikon D70S with Tamron SP AF90mm F/2.8 Di Macro 1:1 lens) and lesion area quantified using computer-assisted imaging analysis with Zeiss Axiovision software (Rel 4.5). Percent-positive area was defined as the fraction of Sudan-positive staining to total area measured.

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Table S1. Pharmacokinetic data following oral dosing of PRT060318

PRT060318 (30 mg/kg in distilled water) or vehicle control were administered via oral gavage twice daily at time 0 and 8 hr later. Plasma concentrations (ng/mL) of the free base of PRT060318 (MW 340.43) were determined at 0, 2, 8, and 10 hr on day 1, day 2, and day 3.

Mouse	Time		PRT060318 plasma concentration	Mean	SD	%CV
	Day	Hour	(ng/mL)			
1	1	0	< 5.00		---	---
2			< 5.00			
3			< 5.00			
4	1	2	2210	1760	404	22.9
5			1430	5.2 µM		
6			1640			
7	1	8	749	819	114	14.0
8			757	2.4 µM		
9			951			
10	1	10	2050	2107	172	8.17
11			2300	6.2 µM		
12			1970			
13	2	0	515	303	184	60.8
14			188	0.9 µM		
15			205			
16	2	2	2180	2243	55.1	2.46
17			2270	6.6 µM		
18			2280			
19	2	8	906	1084	274	25.3
20			1400	3.2 µM		
21			946			
22	2	10	2010	1867	150	8.06
23			1710	5.5 µM		
24			1880			
25	3	0	147	212	61.1	28.8
26			222	0.6 µM		
27			268			
28	3	2	1570	1593	306	19.2
29			1910	4.7 µM		
30			1300			
31	3	8	756	846	151	17.9
32			761	2.5 µM		
33			1020			
34	3	10	2950	2443	594	24.3
35			2590	7.2 µM		
36			1790			
37	24	0	8.86	2.95	5.12	173
38			< 5.00	0.009 µM		
39			< 5.00			

Figure S1. Vascular inflammation and proliferation are attenuated by PRT060318
Quantitative immunohistochemical analysis of femoral arteries 5d after wire injury. Immunostaining for CD45 (A) and BrdU (B). Data represent mean ± SD, n=10 per treatment group.

Figure S2. PRT060318 inhibits the accumulation of neutrophils and macrophages after vascular injury
Quantitative immunohistochemical analysis of femoral arteries 5d after wire injury. Immunostaining for (A) neutrophils (mAb 7/4-positive cells) and (B) macrophages (Mac-3–positive cells). Data represent mean ± SD, n=10 per treatment group.

Figure S3. PRT060318 inhibits arterial Syk activity
Syk kinase activity within the injured vessel wall was examined by assaying for the phosphorylation status of Vav, a downstream Syk target. Phospho-Vav–positive vessel wall (intima and media) area was reduced significantly in PRT060318-treat mice. Data represent mean ± SD, n=7 per treatment group.

Figure S4. PRT060318 administration and mouse body weight
We examined the development of atherosclerotic lesions in ApoE$^{-/-}$ male mice consuming a high-fat diet from 8 to 28 weeks of age. Mice were treated with PRT060318 (30 mg/kg in distilled water, n=15) or vehicle control (n=16) administered via oral gavage twice daily for 3 weeks followed by 1 week off from 8 weeks of age until 24 weeks of age for a total of 16 weeks. Aortas were then harvested at 28 weeks of age for atherosclerotic lesion analysis. Mice were weighed at baseline and weekly thereafter. Data represent mean ± SD.

Figure S1

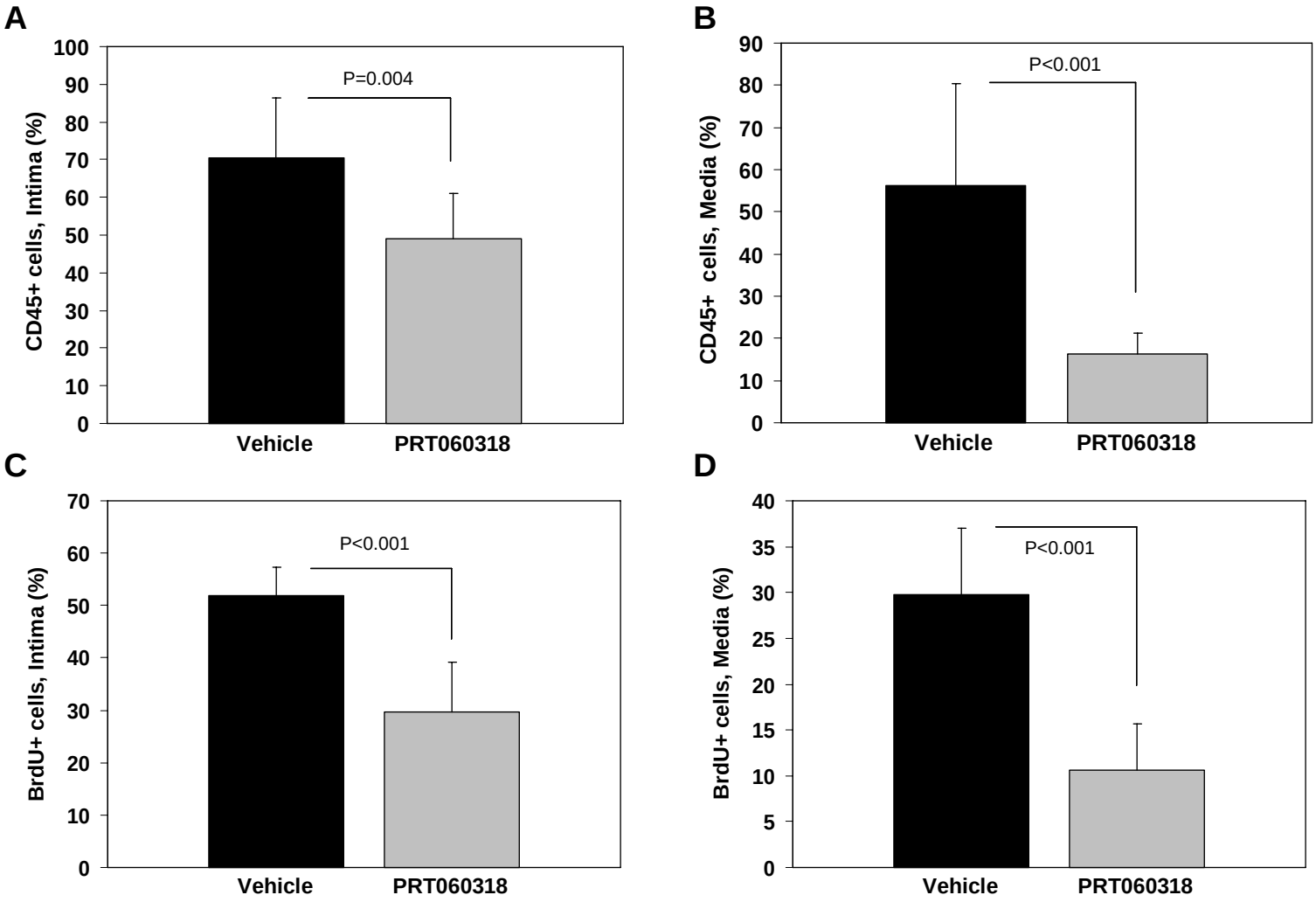

A
CD45+ cells, Intima (%)
P=0.004
Vehicle
PRT060318
B
CD45+ cells, Media (%)
P<0.001
Vehicle
PRT060318
C
BrdU+ cells, Intima (%)
P<0.001
Vehicle
PRT060318
D
BrdU+ cells, Media (%)
P<0.001
Vehicle
PRT060318

Figure S2

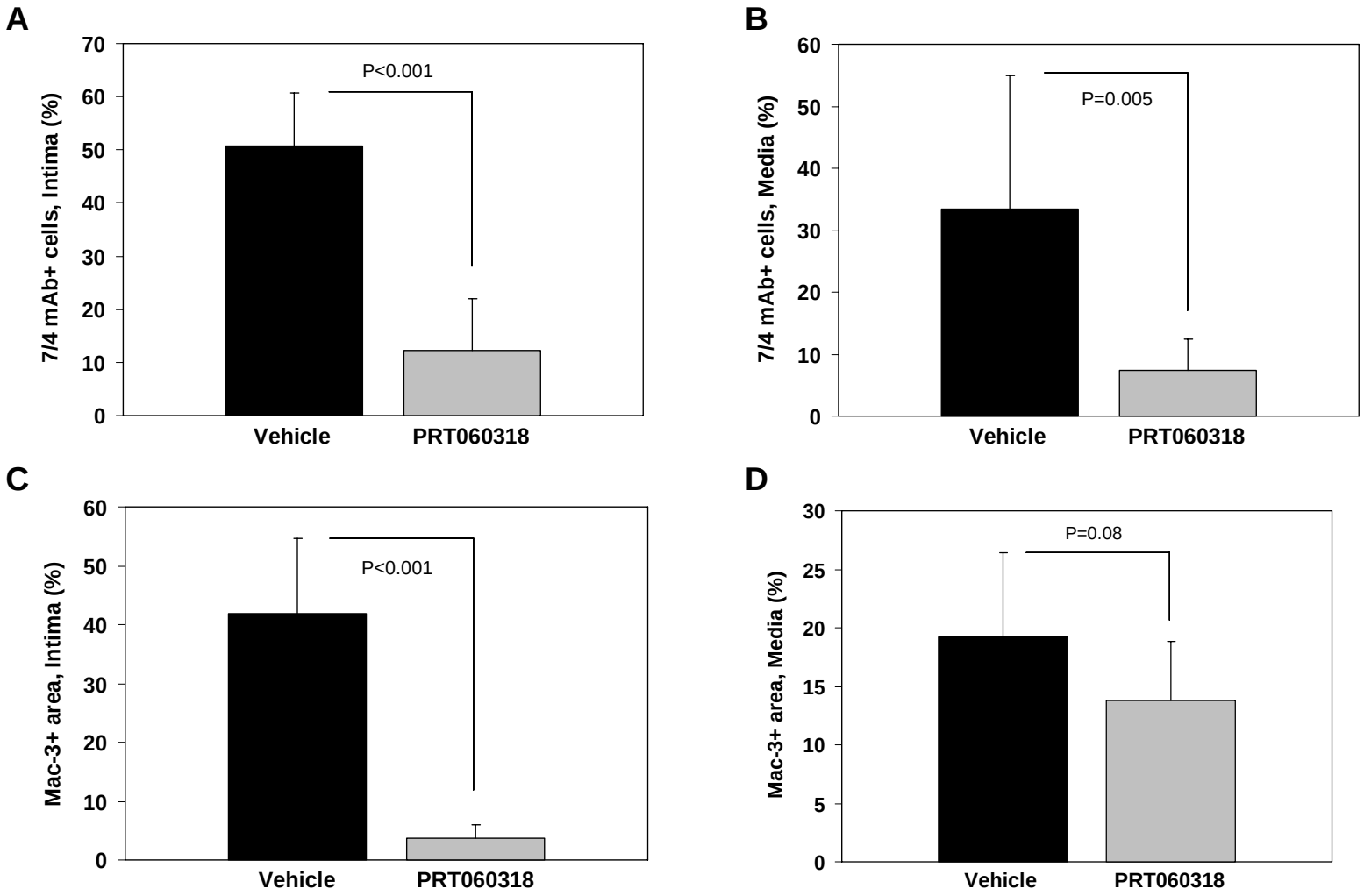
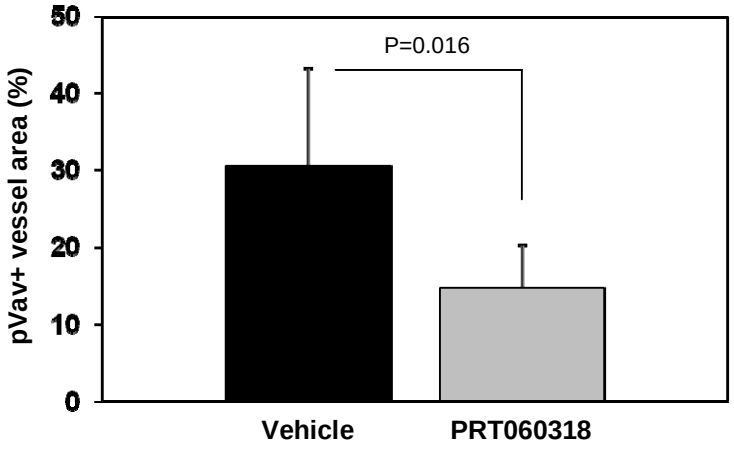

A
7/4 mAb+ cells, Intima (%)
70
60
50
40
30
20
10
0
P<0.001
Vehicle
PRT060318
B
7/4 mAb+ cells, Media (%)
60
50
40
30
20
10
0
P=0.005
Vehicle
PRT060318
C
Mac-3+ area, Intima (%)
60
50
40
30
20
10
0
P<0.001
Vehicle
PRT060318
D
Mac-3+ area, Media (%)
30
25
20
15
10
5
0
P=0.08
Vehicle
PRT060318

Figure S3

A


50
40
30
20
10
0
pVav+ vessel area (%)
P=0.016
Vehicle
PRT060318

B

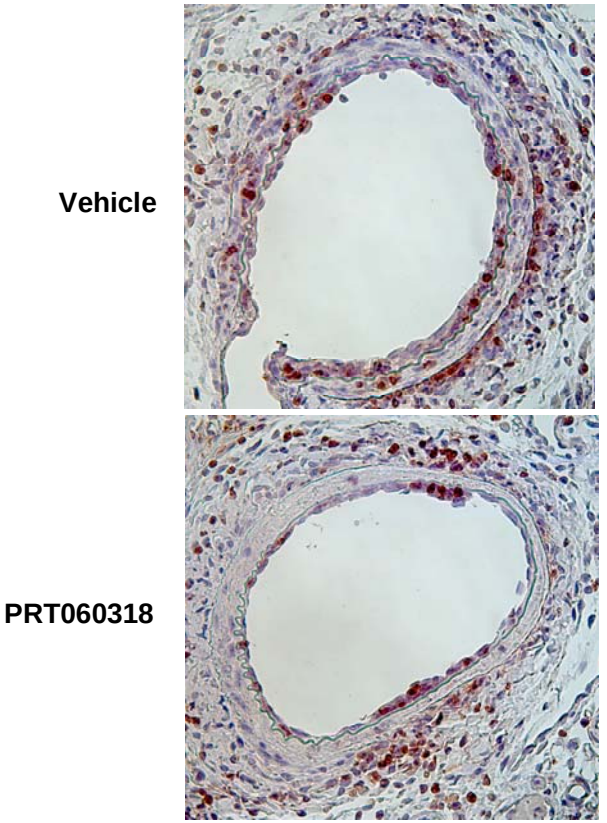
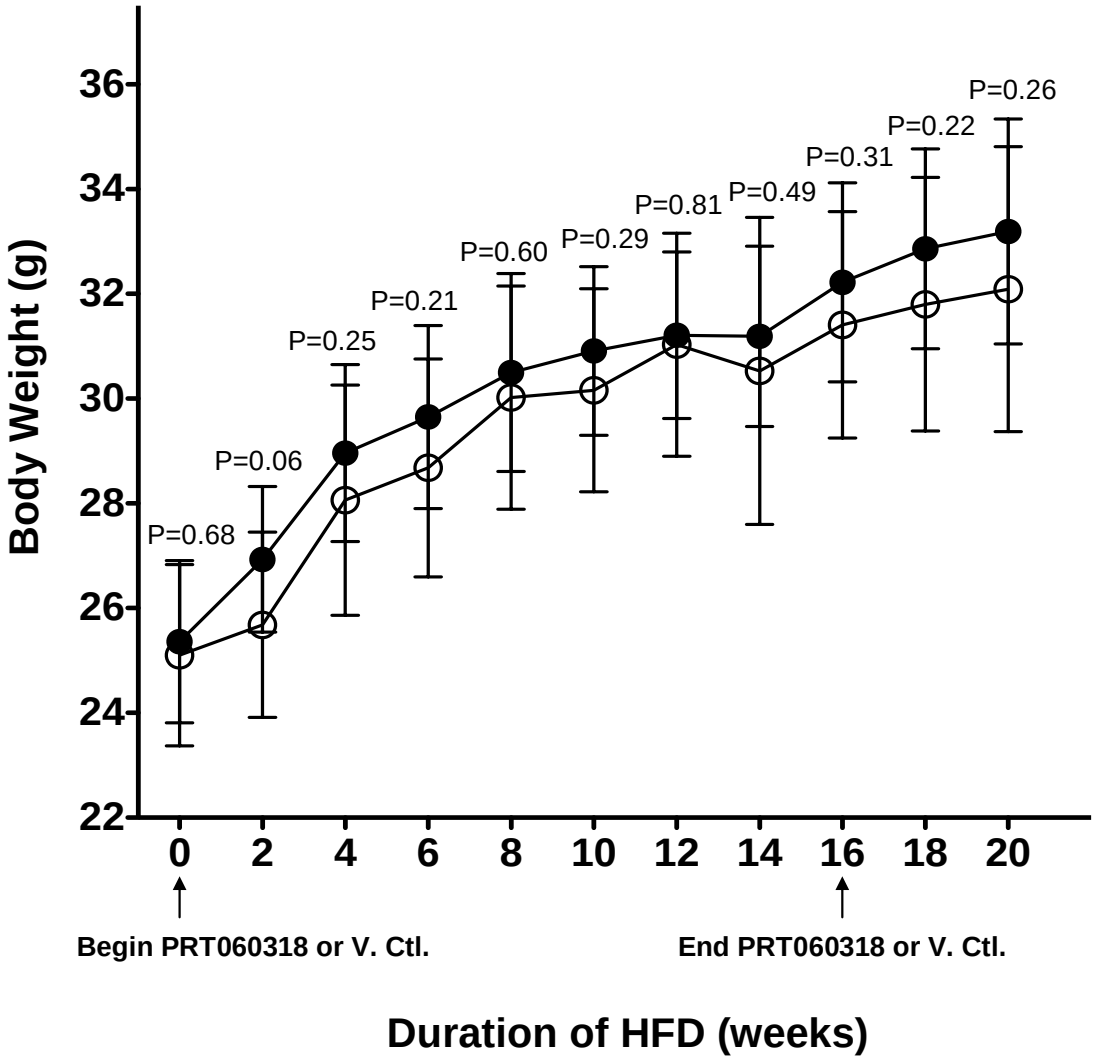

Vehicle
PRT060318

Figure S4


Body Weight (g)
36
34
32
30
28
26
24
22
P=0.68
P=0.06
P=0.25
P=0.21
P=0.60
P=0.29
P=0.81
P=0.49
P=0.31
P=0.22
P=0.26
0
2
4
6
8
10
12
14
16
18
20
Begin PRT060318 or V. Ctl.
End PRT060318 or V. Ctl.
Duration of HFD (weeks)