Materials and Methods

Animals

We generated experimental mice by mating male mice hemizygous for the *Acta2-Cre*ER^{T2} transgene[1] and homozygous for a floxed *Tgfbr2* allele (exon 4);[2] with female mice homozygous for the floxed *Tgfbr2* allele.[3] All experimental mice were *Tgfbr2*$^{flox/flox}$ and either *Acta2-Cre* ER$^{T2\ +/0}$ or *Acta2-Cre* ER$^{T2\ 0/0}$. All alleles were extensively backcrossed (>10 generations) into the C57BL/6 background. We chose the *Acta2-Cre* ER^{T2} allele[1] rather than the *Myh11-Cre* ER^{T2} allele[4] because *Acta2-Cre* ER^{T2} is autosomal, permitting experiments in both male and female mice. Moreover, these 2 alleles have equivalent efficiency and specificity in our hands and in those of others.[5, 6] Mice were housed in a specific-pathogen-free facility, and fed normal chow. Experimental mice were enrolled at 6 weeks of age and euthanized between 9 and 13 weeks of age. Mice of both sexes were enrolled in approximately equal proportions, as further described in Results. All procedures were approved by the University of Washington Office of Animal Welfare.

Genotyping

Mice were genotyped by PCR performed on tail-tip DNA. *Tgfbr2* was genotyped by heating samples to 95°C for 3 minutes, then 37 cycles at 95 °C for 20 seconds, 62 °C for 30 seconds, and 72 °C for 40 seconds. The final step was 72 °C for 7 minutes. Primers were P1: 5'-TATGGACTGGCTGCTTTTGTATTC-3', P2: 5'-TGGGGATAGAGGTAGAAAGACATA-3' and P3: 5'-TATTGGGTGTGGTTGTGGACTTTA-3'. *Acta2-Cre*ER^{T2} was detected by heating samples to 94 °C for 3 minutes, then 43 cycles of 94 °C for 1 minute, 65 °C for 1 minute, and 72 °C for 1 minute. The final step was 72 °C for 5 minutes. Primers were P1: 5´-CCAAAATTTGCCTGCATTACCGGTCGATGC-3´ and P2: 5´-AGCGCCGTAAATCAATCGATGAGTTGCTTC-3´. The R26R allele was detected by heating samples to 94 °C for 3 minutes, then 29 cycles of 94 °C for 30 seconds, 62 °C for 30 seconds, and 68 °C for 1 minute. The final step was 68 °C for 3 minutes. Primers were P1: 5'- AAAGTCGCTCTGAGTTGTTAT-3', P2: 5'-GCGAAGAGTTTGTCCTCAACC-3', and P3: 5'-GGAGCGGGAGAAATGGATATG-3'

Tamoxifen injections and minipump implantation

All experimental mice were injected intraperitoneally with tamoxifen (tamoxifen free base, #T5648; Sigma-Aldrich, St. Louis, MO). Tamoxifen (2.5 mg) was suspended in 250 µL of vehicle (sterile olive oil with 5% ethanol). Injections were begun at 6 weeks of age and continued daily for 5 days. 14 days after the last injection, all mice were implanted with osmotic minipumps (ALZET model 2004, ALZET Osmotic Pumps, Cupertino, CA) containing angiotensin, as described previously.[7] Briefly, angiotensin II (# A9525; Sigma-Aldrich) was equilibrated to room temperature and dissolved in sterile saline to a stock concentration of 10 mg/mL. The stock was further diluted in saline according to the weight of each mouse, lot-specific release rate, and fill volume of the pump so that 1000 ng/kg/min Ang II was delivered for 28 days. The minipumps were loaded according to the manufacturer's instructions and primed in saline for 24 hours at 37 °C. For pump implantation, mice were anesthetized with isoflurane (5% for induction and 1 – 2% for maintenance). Minipumps were implanted subcutaneously on the dorsal neck/upper back.

Blood pressure measurements

Blood pressure was measured noninvasively in non-anesthetized animals, using the CODA Non-Invasive Blood Pressure System (Kent Scientific, Torrington, CT), essentially as described.[8] Mice were placed into a rodent tube holder on a warming platform and allowed to acclimate. When the surface temperature of the tail was 32 – 35 °C, the

occlusion cuff and volume pressure recorder sensor were placed on the tail. Blood pressure was measured on 20 consecutive cycles, with 30 seconds between cycles. Mice in the blood-pressure cohort were all injected with tamoxifen and implanted with Ang II-releasing minipumps at 6 and 8 weeks of age, respectively. Blood pressure was measured 2 and 4 weeks after beginning Ang II infusion.

Injection of TGF-β neutralizing antibody

Hybridoma cells that produce an antibody that neutralizes TGF-β (clone 2G7)[9] were kindly provided by Dr. Ziad Mallat (Cambridge University).[10] Cells were grown in a humidified incubator at 37 °C, 5% CO_2 in a 1:1 mixture of DMEM and F12 medium (#11320-033; Gibco, Thermo Fisher Scientific, Waltham, MA,) with 10% fetal bovine serum. Cells were grown to confluence and fed with serum-free medium. Medium was collected after 48 – 72 hours, loaded on a protein G Sepharose column (# 10-1243 Invitrogen, Thermo Fisher Scientific), and eluted with 0.2 M glycine pH 2.0 into 1/10 volume 1M Tris pH 8.0. Eluted antibody was dialyzed against PBS (pH 7.4), aliquoted, stored at –80 °C and thawed only once (upon use). After the initial dosing study (see below), we injected mice intraperitoneally with 10 mg/kg of this antibody or with the same dose of endotoxin-free mouse IgG (# IR-MS-GF-ED Innovative Research, Novi, MI). Antibody injections were begun on the day of minipump implantation and repeated every 3 days until the mice were euthanized.

Measurement of serum TGF-β1, -β2, and -β3

To test whether the anti-TGF-β antibody clone 2G7 blocked TGF-β1 activity in vivo, we injected mice (n=4 per group, 2 male and 2 female) intraperitoneally with 250 µL of either saline-diluted anti-TGF-β antibody (10, 15, or 20 mg/kg) or saline alone. This was essentially a repeat of a similar experiment reported by Wang et al., an important precedent for our study.[10] Injections were given 3 times, with each injection separated by 3 days. Twenty-four hours after the third injection, mice were deeply anesthetized with ketamine and xylazine, blood was collected by cardiac puncture after thoracotomy, and mice were euthanized by exsanguination. Blood was allowed to clot for 2 hours at room temperature, then centrifuged at 4 °C for 10 minutes (2,040 x g). Serum was collected, aliquoted and stored at -80 °C. Aliquots were thawed only once (upon use). Serum TGF-β1 was measured with an ELISA kit that detects active TGF-β1 (TGF-β1 kit # MB100B, R&D Systems, Minneapolis, MN). To measure total TGF-β1, 40 µL of serum was acidified with 10 µL of 1N HCl for 10 minutes at room temperature and neutralized with 10 µL 1.2 N NaOH, 0.5 M HEPES to a pH between 7.2 and 7.6. Samples were then diluted according to the manufacturer's instructions, and TGF-β1 was measured by ELISA. We selected the 10 mg/kg dose of 2G7 antibody for our study because it produced a significant reduction in serum TGF-β1 in this experiment and because we anticipated that this dose would allow the collection of specimens for histologic analysis. Wang et al reported that a 20 mg/kg dose of 2G7 caused 100% aneurysm prevalence and 80% mortality, whereas a 10 mg/kg dose caused 80% aneurysm prevalence and 40% mortality. Because we aimed to perform tissue analyses on perfusion-fixed aortas, we decided against using the 20 mg/kg dose because—based on Wang et al's results—most of the mice would be expected to die suddenly, precluding proper collection of tissue for analysis.

As our study neared completion, a concern was raised about the ability of the 2G7 antibody to block all isoforms of TGF-β (TGF-β1, -β2 and -β3) in the context of Ang II stimulation. To address this concern, we implanted Ang II pumps in 2 groups of mice (9-10 per group). We treated 1 group with 2G7 (250 µL = 10 mg/kg) and the second group with the same volume and dose of saline-diluted endotoxin-free mouse IgG. Injections were given 3 times, with each injection separated by 3 days. The control group included 5 males and 5 females and the 2G7 group included 6 males and 3 females. Twenty-four

hours after the third injection, mice were deeply anesthetized with ketamine and xylazine, blood was collected by cardiac puncture after thoracotomy, mice were euthanized by exsanguination, and serum was generated, as described above. Total serum TGF-β1 was measured by ELISA (described above) and total TGF-β2 and -β3 were also measured with ELISA kits (TGF-β2 kit #MB200 R&D Systems, Minneapolis, MI; TGF-β3 kit # EK1104; Boster Biological Technology Co, Pleasanton, CA) according to the manufacturer's instructions.

In vitro testing of anti-TGF-β antibody clones 2G7 and 1D11

To test whether TGF-β1, -β2, and -β3 are inhibited in vitro by anti-TGF-β antibodies commonly used in vascular research [clones 1D11 (R&D Systems) and 2G7], 250 pg/mL of recombinant TGF-β1, -β2 or -β3 (supplied with the ELISA kits listed above) were incubated without or with increasing concentrations (1, 10, 30, 100, and 300 μg/mL) of 2G7 antibody, 1D11 antibody, or control mouse IgG for 90 minutes at room temperature. ELISAs for all three TGF-β isoforms were performed according to the manufacturer's instructions.

Tissue fixation and storage

Mice in the aneurysm study (Figure 1) were deeply anesthetized and exsanguinated by saline perfusion. Aortas were then fixed in situ by perfusion at physiologic pressure with 10% formalin, excised along with the attached heart, incubated in 10% formalin overnight, and stored at 4 °C in 70% ethanol. Adipose tissue surrounding the aorta and heart was removed using forceps and a stereomicroscope (Wild M3Z, Leica Microsystems, Buffalo Grove, IL). The hearts and aortas were removed from 70% ethanol and photographed in a dissection dish (#DD-90-S; Living Systems Instrumentation, St. Albans, VT) using a digital camera attached to the stereomicroscope (model DFC295, Leica Microsystems).

Measurement of maximal abdominal aortic diameter

Explanted aortas were fixed to the bottom of a silicone-covered dissection dish by placement of pins in the heart and great vessels. The distal aorta was then rotated as needed so that the maximal diameter of the abdominal aorta was parallel to the bottom of the dish. Pins were inserted to fix the specimen to the dish in this position. The abdominal aorta was then photographed with a ruler alongside. This image was later used, with computer-assisted planimetry, to measure the maximal external diameter of the abdominal aorta. The maximal aortic diameter was measured as the distance from one edge of the adventitia to the opposite edge. An observer, blinded to the experimental groups, made this measurement at the site of maximal dilation, in a plane orthogonal to the major aortic axis at this site; the location of this site varied among animals. Because of a technical error made during specimen preparation, one mouse in the SMC-specific TBRII loss group was excluded from maximal abdominal aortic diameter measurement. To assess the inter-observer reproducibility of maximal external diameter measurements, we assigned a second observer to re-measure the diameter in a group of 38 randomly chosen abdominal aortas. The range of these measurements was 0.9–4.5 mm. The mean difference between measurements obtained by the first and second observers was 0.03 mm with a standard deviation of 0.11 mm. Over 94% of measurements made by the 2 observers were within the limits of agreement, as defined by Bland and Altman.[11]

Macroscopic assessment of abdominal aortic pathology

Images of explanted abdominal aortas were evaluated by three independent observers, blinded to experimental group. The observers also had access to the fixed aortic specimens. If an observer deemed an image ambiguous, the actual specimen was examined. The observers assessed the presence of abdominal aortic pathology (e.g.,

either aortic dilation or apparent intramural blood), and assigned a severity grade to each aorta. Severity grades were assigned by each observer according to a scale,[12] with modifications that included addition of a grade of "0" for aortas with no visible aneurysmal pathology (Figure 2E). Most grades were identical among the 3 observers. When grades differed, we used the median as the final grade. For grading of abdominal aortic pathology there was agreement between at least two observers in 68 of 72 specimens (94%). To further assess agreement among abdominal aortic aneurysm severity scores assigned by different observers, we calculated the weighted kappa values for the three pairs of observers.[13] These values ranged between 0.7 and 0.8, indicating moderate to strong agreement. Weighted kappa values comparing the scores of each of the 3 observers to the final median score were 0.8–1.0. These data suggest that our abdominal aortic pathology severity scoring system is reproducible and that the final median score for each aorta agrees strongly with the scores of each of the 3 observers.

Measurement of ascending aortic external diameter

We measured external diameters of the ascending aortas using en bloc excised specimens of heart and aorta. We removed the atria from these specimens, exposing the proximal aorta. The specimens were then placed on the bottom of a silicone-covered glass dish. The heart and aorta were fixed to the dish by insertion of pins into the heart and one of the great vessels. As a result, the aorta was apposed to the dish along its entire length, eliminating the antero-posterior curvature of the ascending aorta and arch. A ruler was placed adjacent to the aorta and was imaged along with the thoracic aorta. This image was later used, with computer-assisted planimetry, to measure the external diameter of the aorta in a plane orthogonal to the major aortic axis. This plane, just proximal to the takeoff of the innominate artery, is the same location as "Cut 2", shown on Supplemental Figure III. Unlike the abdominal aortas, in which dilation was evident in many specimens but varied in location, there was no evident dilation of the thoracic aortas. For this reason, we decided to measure all of the thoracic aortas at the single standardized location described above. Because of a technical error made during specimen preparation, one mouse in the SMC-specific TBRII loss group was excluded from all thoracic aortic assessments.

Macroscopic assessment of thoracic aortic pathology

Images of explanted thoracic aortas were evaluated by the same three observers. Because macroscopic pathology in the thoracic aorta was limited to apparent aortic wall hematoma, we developed a grading system to assess hematoma severity (Figure 3D). When grades differed, we used the median as the final grade. For grading of thoracic aorta pathology there was agreement between at least two of the three observers in 69 of 70 specimens (99%). To further assess agreement between thoracic aortic hematoma severity scores assigned by different observers, we calculated the weighted kappa values for the three pairs of observers.[13] These values ranged from 0.8 to 0.9, indicating strong agreement. Weighted kappa values comparing the scores of each of the 3 observers to the final median score also ranged from 0.8 to 0.9. These data suggest that our thoracic aortic hematoma severity scoring system is reproducible and that the final median score for each aorta agrees strongly with the scores of each of the 3 observers

Tissue processing, sectioning and staining

We used random number generation (random.org) to select a subset of 12 aortas from each of the 3 groups for histologic analyses. The heart was detached from the aorta by transverse sectioning of the aorta as close to the heart as possible. The aortas were then sectioned transversely at a point halfway between the caudal end of the lesser curvature and a point 0.5 mm cranial to the superior mesenteric artery takeoff (cut 4 in

Figure III in the online-only Data Supplement). This segment of thoracic aorta was sectioned transversely (cuts 2 and 3 in Figure III in the online-only Data Supplement) into 3 smaller segments. These 3 segments were then trimmed to yield 3 smaller segments (indicated by rectangles in Figure III in the online-only Data Supplement). These 3 smaller segments—containing tissue from the ascending thoracic aorta (AscA), the aortic arch (AR) and the cranial portion of the descending thoracic aorta (cDTA)—were embedded alongside each other in a single block of optimal cutting temperature (OCT) compound (#4583; Sacura Finetek USA, Torrance, CA). For all 3 segments, the edge closest to the aortic valve was placed at the block face at which sectioning was begun.

The caudal portion of the aorta (below cut 4 in Figure III in the online-only Data Supplement) was cut transversely 0.5 mm cranial to the superior mesenteric artery takeoff and halfway between the left renal artery and the bifurcation (cuts 5 and 6 in Figure III in the online-only Data Supplement). The most distal segment (caudal infrarenal aorta) was discarded. The 2 remaining segments contained: 1) the distal descending thoracic aorta and part of the suprarenal abdominal aorta; and 2) the remainder of the suprarenal abdominal aorta, the juxtarenal aorta, and the most cranial part of the infrarenal aorta. These 2 segments were embedded in a single OCT block alongside each other. For both segments, the edge created by the cut near the SMA takeoff (cut 5 in Figure III in the online-only Data Supplement) was placed at the block face at which sectioning was begun. When sectioned at 5 steps in both directions, the first 2 steps in both directions were termed cranial abdominal aorta (cAA). The 3 most cranial sections were termed distal descending thoracic aorta (dDTA), and the 3 most caudal sections were termed infrarenal aorta (IR).

The block containing the AscA, AR, and cDTA was sectioned at 3 steps, each separated by 150 μm. At each of the 3 steps we cut 12 serial 5-μm-thick sections (3 segments, 3 steps per segment; 12 sections per step = 108 sections with 3 sections per segment = 36 slides). The block containing the dDTA, the cAA, and the IR was sectioned at 5 steps, each separated by 1.9 mm. At each of the 5 steps, we cut 20 serial 5-μm-thick sections (2 segments, 5 steps per segment, 20 sections per step = 200 sections with 2 sections per segment = 100 slides). Sections were mounted on glass slides (#48311-703; VWR, Randor, PA), and stored at –80 °C.

All histologic sections were cut in planes as close to orthogonal as possible in relation to the major aortic axis. With reference to Supplemental Figure III, segments of cDTA, dDTA, cAA, and infrarenal aorta were all sectioned orthogonally to the lumen. Segments of the ascending aorta (AscA) and arch (AR) were slightly curved; therefore, some sections are cut at small angles to the orthogonal plane. These small angles were likely consistent among all of the aortas, minimizing opportunity for bias.

For hematoxylin and eosin stain, slides were incubated at room temperature for 1 hour, then stained with eosin y solution (#HT110132-1L; Sigma Aldrich) diluted 1:20 in deionized water, and counterstained with Gill's hematoxylin solution (#CS400-D; Thermo-Fisher Scientific). Slides were then washed, dehydrated (95% ethanol), covered using Permount medium (#SP15-500; Thermo-Fisher Scientific).

Slides were permeabilized with acetone for 10 minutes at –20 °C, washed with cold PBS, and blocked with 3% solution (w/v) of bovine serum albumin in PBS. After washing in PBS, slides were incubated overnight at 4 °C with Mac-2 antibody (anti-galectin 3; diluted 1:500 in 3% BSA solution; #CL8942AP, Cedarlane Labs, Burlington, NC). Negative controls were stained with nonimmune Rat IgG2b (#MCA6006; AbD Serotech, Kidlington, UK;) or no primary antibody. Bound antibody was detected with

the Vectastain ABC and Nova Red Peroxidase substrate kits (#PK 4000 and #SK-4800; Vector Labs, Burlingame, CA). Slides were covered using Permount medium, and stored at room temperature.

Stained slides were imaged using a microscope and camera (DM4000 B microscope, DFC295 camera; Leica Microsystems) and images were analyzed using the Image J software (version 1.48, NIH, Bethesda, MD). Some of the images were converted to 8-bit format, background signal was removed by thresholding, and the area above threshold was measured.

Planimetry

Hematoxylin and eosin-stained sections were illuminated through a fluorescein filter (Leica Microsystems) to induce autofluorescence of the elastic laminae, and imaged as described above. An observer blinded to experimental group used Image J software to measure the circumferences of the internal and external elastic laminae (IEL and EEL, which are essentially the lumenal and outer medial borders) and the outer perimeter of the adventitia. We calculated average medial thickness, adventitial thickness and lumen diameter as previously described,[14] by assuming circular in vivo geometry of the IEL, EEL and outer adventitial circumferences and calculating the differences between the radii of these circles. To check for inter-observer reproducibility of planimetry measurements, a second observer re-measured the IEL, EEL, and outer adventitial circumferences of a subset of randomly chosen sections. Measurements of thoracic aortic sections ranged from 2.7–4.4 mm (includes measurements of IEL, EEL, and outer adventitial circumferences), with a mean difference between measurements from the two observers of 0.06 mm. The standard deviation of these differences was 0.14 mm (n=24). Measurements made on abdominal aortic sections ranged from 2.4–5.0 mm (again includes measurements of IEL, EEL, outer adventitial circumferences). The mean difference between measurements of the two observers was 0.07 mm, and the standard deviation of these differences was 0.19 mm (n=120). For both thoracic and abdominal aortic sections, >94% of measurements made by the 2 observers were within the limits of agreement, as defined by Bland and Altman.[11]

Aortic protein isolation

Mice were deeply anesthetized with ketamine and xylazine and exsanguinated by saline perfusion. Aortas were excised, trimmed of periaortic fat, snap-frozen and stored at –80 °C. The aorta was later thawed, opened longitudinally, and pinned lumen-up on a clear dissection dish. The endothelial layer was scraped away with a cotton swab, a transverse incision was made through the media with a scalpel, and the medial and adventitial layers separated with forceps. The media was then placed in liquid nitrogen, crushed with a liquid nitrogen-chilled steel mortar and pestle, and incubated on ice with 100 µL of complete lysis buffer (#04719956001; Roche Applied Sciences, Penzberg, Germany) for 30 minutes. The sample was spun at 16,100 x g and the amount of protein in the supernatant measured with the BCA assay (#23227; Thermo-Pierce).

Western blotting

For each sample, 7 µg of aortic medial protein was separated with SDS/PAGE (#NP0335; Invitrogen, Waltham, MA) and transferred to a PVDF membrane (# IPSN07852; EMD Millipore, Billerica, MA). TBRII was detected with a rabbit primary antibody (#SC-4001; 1:1000 dilution; Santa Cruz Antibodies, Santa Cruz, CA) and an HRP-conjugated anti-rabbit secondary antibody (#170-6515; 1:2000 dilution, Bio Rad, Hercules, CA). To control for protein loading and transfer, membranes were stripped and re-probed for either β-actin (#A5316; 1:2000 dilution; Sigma-Aldrich) or GAPDH (#SC-20357; 1:1000 dilution; Santa Cruz Antibodies), followed by application of HRP-

conjugated antibodies to mouse IgG (#170-6516; 1:2000 dilution; Bio Rad) or goat IgG (#SC-203576; 1:2000 dilution; Santa Cruz Antibodies), respectively.

Statistics

All statistical analyses were carried out with the SigmaStat software (version 13, Systat Software Inc., San Jose, CA). Survival curves were compared with the log-rank test. Categorical data (prevalence of aortic pathology) were compared using the chi-square RxC contingency table test.[15] Ordinal data (severity of aortic pathology) were compared using the chi-square test. In experiments with 2 groups (TBRII expression levels and antibody-mediated blockade of TGF-β), continuous data were compared by t-test after confirming normality and equal variances of the groups. In the first experiment testing whether increasing doses of anti-TGF-β antibody (clone 2G7) reduced the amount of serum TGF-β, we used a one-tailed t-test because we anticipated (from previous studies and our own preliminary in vitro experiments) the direction of the effect. In later experiments testing whether 2G7 blocks TGF-β1, -β2, and -β3, we used a two-tailed t-test. In experiments with 3 groups and continuous data we used one-way analysis of variance (ANOVA). If the P value was < 0.05 we used Dunnett's test to compare each of the 2 experimental groups to the control group. Group data are shown as mean ± SEM. Continuous data that failed to meet the criteria for normal distribution and equal variance were analyzed using the Kruskal-Wallis one-way ANOVA on ranks test. If the P value of this ANOVA was less than 0.05, each of the two experimental groups was compared to the control group using Dunn's method for post-hoc correction for pairwise comparisons. Inter-observer reproducibility of IEL, EEL and outer adventitial circumference measurements was assessed by calculating the mean difference and limits of agreement for measurements made by two observers.[11] Reproducibility of observers' thoracic and abdominal aortic pathology severity scores was assessed by calculating weighted kappa scores for each pair of observers and for each observer's scores compared to the final median score.[13]

References for Materials and Methods

1. Wendling O, Bornert JM, Chambon P, Metzger D. Efficient temporally-controlled targeted mutagenesis in smooth muscle cells of the adult mouse. *Genesis*. 2009;**47**:14–18
2. Leveen P, Larsson J, Ehinger M, Cilio CM, Sundler M, Sjostrand LJ, Holmdahl R, Karlsson S. Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood*. 2002;**100**:560–568
3. Soriano P. Generalized lacz expression with the rosa26 cre reporter strain. *Nat. Genet.* 1999;**21**:70–71
4. Wirth A, Benyo Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, Orsy P, Horvath B, Maser-Gluth C, Greiner E, Lemmer B, Schutz G, Gutkind JS, Offermanns S. G12-g13-larg-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat. Med.* 2008;**14**:64–68
5. Wei H, Hu JH, Angelov SN, Fox K, Yan J, Enstrom R, Smith A, Dichek DA. Aortopathy in a mouse model of marfan syndrome is not mediated by altered transforming growth factor beta signaling. *J Am Heart Assoc*. 2017;**6**
6. Herring BP, Hoggatt AM, Burlak C, Offermanns S. Previously differentiated medial vascular smooth muscle cells contribute to neointima formation following vascular injury. *Vasc Cell*. 2014;**6**:21

7. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein e–deficient mice. *J. Clin. Invest.* 2000;**105**:1605–1612
8. King VL, Lin AY, Kristo F, Anderson TJ, Ahluwalia N, Hardy GJ, Owens AP, 3rd, Howatt DA, Shen D, Tager AM, Luster AD, Daugherty A, Gerszten RE. Interferon-gamma and the interferon-inducible chemokine cxcl10 protect against aneurysm formation and rupture. *Circulation*. 2009;**119**:426–435
9. Lucas C, Bald LN, Fendly BM, Mora-Worms M, Figari IS, Patzer EJ, Palladino MA. The autocrine production of transforming growth factor-beta 1 during lymphocyte activation. A study with a monoclonal antibody-based elisa. *J. Immunol.* 1990;**145**:1415–1422
10. Wang Y, Ait-Oufella H, Herbin O, Bonnin P, Ramkhelawon B, Taleb S, Huang J, Offenstadt G, Combadiere C, Renia L, Johnson JL, Tharaux PL, Tedgui A, Mallat Z. Tgf-beta activity protects against inflammatory aortic aneurysm progression and complications in angiotensin ii-infused mice. *J. Clin. Invest.* 2010;**120**:422–432
11. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*. 1986;**1**:307-310
12. Daugherty A, Manning MW, Cassis LA. Antagonism of at2 receptors augments angiotensin II-induced abdominal aortic aneurysms and atherosclerosis. *Br. J. Pharmacol.* 2001;**134**:865–870
13. Cohen J. A coefficient of agreement for nominal scales. *Educational and Psychological Measurement*. 1960;**20**:37–46
14. Hu JH, Wei H, Jaffe M, Airhart N, Du L, Angelov SN, Yan J, Allen JK, Kang I, Wight TN, Fox K, Smith A, Enstrom R, Dichek DA. Postnatal deletion of the type II transforming growth factor-beta receptor in smooth muscle cells causes severe aortopathy in mice. *Arterioscler. Thromb. Vasc. Biol.* 2015;**35**:2647–2656
15. Armitage P, Berry G. *Statistical methods in medical research*. Oxford, UK: Blackwell; 1994.