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Quantitative Estimates of the Variability of In Vivo Sonographic Measurements of the Mouse Aorta for Studies of Abdominal Aortic Aneurysms and Related Arterial Diseases

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Abstract

Objectives—Burgeoning interest in reducing the morbidity and mortality associated with abdominal aortic aneurysms (AAAs) has led to experimental strategies to elucidate the disease process and attain pharmacologic regression using the apolipoprotein E^{-/-} (ApoE^{-/-}) mouse model of angiotensin-induced AAAs and in vivo sonography. However, the variability of in vivo sonographic measurements of the mouse aorta has not been established. Thus, our purpose was to determine quantitative estimates of the variability of in vivo sonographic measurements of the mouse aorta as a guide for the design and assessment of studies focused on regression of AAAs and related arterial diseases.

Methods—We used Bland-Altman, locally weighted scatterplot-smoothing regression, and resampling (bootstrapping) methods for variability analyses of multiple in vivo short- and long-

axis sonographic measurements of ApoE^{-/-} mouse aortas. We measured distinct aortic sites in vivo at the baseline and after angiotensin-induced AAAs and ex vivo using digital calipers.

Results—We analyzed 236 data points from 10 male mice (14 weeks old; mean weight $\pm$ SD, 29.7 $\pm$ 1.6 g). Overall intramouse differences between short- and long-axis and in vivo and ex vivo measurements were 0.038 (95% confidence interval [CI], 0.031–0.046) and 0.085 (95% CI, 0.062–0.109) mm, respectively. Intermouse differences in short-axis measurements were 0.047 (95% CI, 0.042–0.053), 0.049 (95% CI, 0.044–0.055), and 0.039 (95% CI, 0.036–0.042) mm for infrarenal, suprarenal, and thoracic measurements, respectively; differences in long-axis measurements were 0.054 (95% CI, 0.044–0.064), 0.029 (95% CI, 0.024–0.034), and 0.046 (95% CI, 0.037–0.054) mm. Bland-Altman and locally weighted scatterplot-smoothing analyses showed excellent agreement between measures with no variation in discrepancies vis-à-vis the target measurement.

Conclusions—These data establish previously undefined estimates of measurement variability relevant for in vivo sonographic studies of AAA regression in a commonly studied mouse model.

Keywords

abdominal aortic aneurysm; mouse model; sonography; variability

Subcutaneous infusion of angiotensin II (AngII) causes the development of abdominal aortic aneurysms (AAAs) in genetically modified mice with hypercholesterolemia.^{1–6} Thus far, experimental strategies to elucidate disease processes and therapeutic interventions have relied almost exclusively on direct anatomical evaluation. However, with advances in imaging technology, imaging agent chemistry, and molecular biology, in vivo evaluation of disease processes in animal models is now feasible.

Noninvasive imaging technologies such as high-frequency microsonography are emerging methods for in vivo characterization of atherosclerosis and AAA disease mechanisms in small-animal models. Other microimaging modalities include magnetic resonance imaging, computed tomography, single-photon emission computed tomography, and positron emission tomography. However, ultrasound systems are more widespread in the in vivo imaging arena because they are portable, are devoid of the potential confounding effects of radiation, obviate the need for dedicated facilities, are easy to use, and are cost-effective compared to other microimaging modalities. In addition, high frame rates provide the best temporal and spatial resolution compared to other modalities.

Sonography affords real-time effectiveness to monitor and quantify blood flow, measure the vessel size and thickness, and evaluate rapid physiologic processes. Furthermore, imaging resolution of up to 30 μ m⁷ allows for delineation of tiny vasculature as well as molecular interrogation of disease process aided by contrast microbubbles labeled with molecular probes. Consequently, an understanding of the reliability of sonographic technology is crucial for repetitive surveillance and assessment of the efficacy of novel interventions for AAA regression. In this regard, there have been reports demonstrating the use of sonography for in vivo visualization and measurement of AAAs. The first report of noninvasive sonographic evaluation of AAAs was by Wang et al⁵; subsequently, additional studies of in vivo sonography of AAAs have been documented.^{8–12} However, to our knowledge, only 2 studies^{10,11} have attempted to assess the agreement between measurements derived from sonography. These studies reported a high correlation between sonographic and morphometric measurements and small intraobserver and interobserver variance. However, correlation coefficients address the strength of relationships rather than agreement between measurements and do not provide insight on precision of measurements.^{13,14} Thus,

easily interpretable and biologically relevant quantitative estimates of measurement variability have been lacking.

We have addressed these issues by using Bland-Altman, nonparametric regression, and resampling methods for analyses of the variability of multiple *in vivo* sonographic measurements of apolipoprotein E^{-/-} (ApoE^{-/-}) mouse aortas. We derived robust precision estimates and discuss our results in the context of the biological relevance and implications for current efforts targeted at regression of AAAs, atherosclerosis, and related arterial disease.

Materials and Methods

Mice

Ten 14-week-old male ApoE^{-/-} mice on a C57BL/6 background were acquired from The Jackson Laboratory (JAX Mice and Services, Bar Harbor, ME) and maintained with a normal mouse chow diet (RP5015; PMI Feeds, Inc, St Louis, MO). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University Institutional Animal Care and Use Committee and the National Institutes of Health.

Sonography and AngII Infusion

Animal Preparation—A high-resolution sonographic microimaging system (Vevo 770; VisualSonics, Inc, Toronto, Ontario, Canada) was used. Mice were anesthetized in a chamber using isoflurane, 2.0% to 2.5% for induction and 1.25% to 1.75% for maintenance. Anesthetized mice were then secured in a supine position onto a Vevo mouse-handling platform with limbs taped onto temperature- and heart rate-monitoring electrodes. Mice were shaved from the thorax to the lower abdominal region using a depilatory cream (Nair; Church & Dwight Co, Inc, Princeton, NJ). Prewarmed ultrasound transmission gel (Aquasonic 100; Parker Laboratories, Inc, Fairfield, NJ) was applied onto the shaved region of interest, creating a coupling medium for transmission of ultrasonic signals between the transducer and animal.

Image Acquisition—Mice underwent a 6- to 7-hour fast before repeated B-mode (2-dimensional) short- and long-axis cine loops of the aorta were acquired using a real-time microvisualization transducer (RMV-704; VisualSonics, Inc) at a central frequency of 40 MHz, a focal length of 6 mm, a field of view of 10 × 10 mm, and a frame rate of 34 Hz. After baseline imaging (3 times over 3 days, once per day, in random order), mice underwent infusion of AngII (catalog number A9525; Sigma-Aldrich, Inc, St Louis, MO; 1500 ng/kg/min), within a 4-week period, as previously described,³ via subcutaneously implanted osmotic pumps (Alzet model 2004; Durect Corporation, Cupertino, CA) followed by repeated sonography before euthanasia and *ex vivo* evaluation.

Determination of Aortic Diameters

Aortic diameters were determined 3 times in the short-axis orientation at the iliac, infrarenal, right renal, suprarenal, and thoracic anatomic locations; corresponding dimensions in the long-axis orientation were determined only at the infrarenal, suprarenal, and thoracic sites. The second set of sonographic measurements was taken at the end of AngII infusion to determine the presence and size of the AAA before euthanasia for direct *ex vivo* determination of aortic diameters. On detection of an aneurysm, the maximal short-axis diameter was documented along with the corresponding long-axis diameter. Pre-euthanasia and *ex vivo* diameters were determined once at the iliac, infrarenal, suprarenal, and thoracic locations. All sonographic aortic diameters were determined by measuring between the

luminal walls of the aorta. Two-dimensional guided power and spectral Doppler patterns aided target vessel confirmation. Image analyses were done offline on digitally stored recorded cine loops at variable frame rates of 1 to 100 Hz using the built-in software analysis package of the Vevo 770 system.

Gross Evaluation

On termination of AngII infusion, the abdominal and thoracic cavities were opened to expose the aorta, which was then perfused through the left ventricle using phosphate-buffered saline introduced via a right atrial incision. With the aid of a dissecting microscope (VanGuard; VEE GEE Scientific, Inc, Kirkland, WA), the heart and attached aorta including the iliac bifurcation were extracted. The aorta was then separated from the heart 2 mm below the aortic arch, fixed in 4% paraformaldehyde for 24 hours, and embedded in paraffin. Before fixation, ex vivo diameters of the iliac, infrarenal, suprarenal, and thoracic locations were measured using digital calipers (Tresna; Guilin Guanglu Measuring Instrument Co, Ltd, Guilin, China) under microscopic guidance.

Immunohistologic Assessment

Immunohistochemical staining was performed to assess the presence of macrophages, elastin degradation, and vascular smooth muscle cells. Embedded tissue was cut into 3- μ m-thick serial sections and stained for hematoxylin and elastic van Gieson. Sections were deparaffinized before being stained with Weigert's iron hematoxylin for 15 minutes, followed by a van Gieson stain for 5 minutes after washing with running water. Formaldehyde-fixed paraffin-embedded sections were deparaffinized by placing sections at 70°C for 1 hour followed by rehydration to water using several changes of xylene and ethanol. When required, heat-mediated antigen retrieval with 10-mmol/L citrate buffer, pH 6, was done for 30 minutes as well as quenching of endogenous peroxidase by submerging sections in 3% hydrogen peroxide for 15 minutes. Potential nonspecific sites were blocked using normal serum for 30 minutes. Slides were incubated with a primary antibody at room temperature for 1 hour (F4/80 [macrophages]; (Gene-Tex, Inc, Irvine, CA) or at 4°C overnight (CD3; Abcam PLC, Cambridge, MA). Negative controls were prepared without a primary antibody to check for nonspecific staining. Anti-mouse, anti-rat, and anti-rabbit Vectastain Elite ABC kits (Vector Laboratories, Inc, Burlingame, CA) were used for the secondary biotinylated antibody and streptavidin-peroxidase complex. A Vector Nova Red substrate kit was used as a substrate for the peroxidase; if counter-stained, Gills hematoxylin III (EMD Chemicals, Gibbstown, NJ) was used. Slides were simultaneously stained and quantified by 2 investigators. A VanGuard 1490-FLP01 microscope (VEE GEE Scientific, Inc) with a mounted camera (EOS Rebel X Si; Canon USA, Inc, Lake Success, NY) was used for analysis of slides and image capture. Stains for macrophages were analyzed using stereology. For this procedure, a microscope with a mounted camera (BX50W1; Olympus Optical Co, Inc, Tokyo, Japan) and stereology software (Stereologer 2000; SRC, Inc, Chester, MD) was used.

Statistical Analyses

We conducted multiple analyses of variability: intramouse in vivo short- versus long-axis measurements, pre-euthanasia versus ex vivo diameters, intramouse variability of repeated baseline in vivo short-axis measurements, and intermouse variability of short- and long-axis in vivo diameters. We assessed agreement between measurement pairs of interest using the Bland-Altman method.¹⁴ Locally weighted scatterplot-smoothing^{15,16} regression was applied for non-parametric evaluation of the local relationship between measurements of interest across parts of their ranges. Locally weighted scatterplot-smoothing trend lines are superimposed on all Bland-Altman plots. For all analyses of variability, a quantitative estimate was determined by calculating the mean absolute differences between

measurements of interest, and then bootstrapping—a modern, nonparametric, computer-intensive resampling method—was applied (using 10,000 random samples with replacement from the sample of mice) to derive robust estimates of the confidence limits for all mean absolute differences. All analyses and graphic illustrations were performed using the R statistical package.¹⁷ All aortic diameter measurements and quantitative estimates of variability are reported in millimeters.

Results

The mice had a mean weight of 29.7 g (range, 27.5–31.9 g). Baseline in vivo measurements of mouse aortas were collected at 5 locations (short axis) 3 times per mouse and at 3 locations (long axis) only once per mouse, thus corresponding to 15 short- and 3 long-axis measurements per mouse and 150 short- and 30 long-axis measurements for 10 mice, totaling 180 baseline data points (Table 1). Similarly, pre-euthanasia in vivo and ex vivo measurements were collected at 4 locations, respectively, for each of the 7 mice that completed the experiment, corresponding to 56 data points (Table 2). All 236 data points were used for analyses.

In Vivo Imaging and Ex Vivo Evaluation of Aneurysmal and Nonaneurysmal Aortas

We used a 2-dimensional sonographically guided spectral Doppler technique to confirm the identity of the target vessel. Representative spectral Doppler images of the aorta, vena cava, and a nonvascular focus show a distinct pulsatile spectral pattern of the aorta due to contraction and relaxation of the heart, in contrast with a less-pulsatile pattern in the vena cava and a baseline undulating spectral signal in the nonvascular focus suggestive of respiratory or other non-blood flow-related motion (Figure 1A). Representative short-axis images delineating the iliac (postiliac bifurcation), infrarenal, right renal (takeoff of the right renal artery), suprarenal, and thoracic aorta are shown in Figure 1B. Corresponding complete long-axis images of the infrarenal, suprarenal, and thoracic aorta are shown in Figure 1C. After AngII infusion, all mice developed an AAA. Three mice died from necropsy-confirmed AAA rupture, whereas the 7 surviving mice had an AAA as detected by in vivo sonography before euthanasia. Representative short-axis images of infrarenal, suprarenal, and thoracic aortas with corresponding long-axis images of the suprarenal aorta and gross anatomic images of the aneurysmal aorta provide in vivo and ex vivo confirmation of presence of an AAA (Figure 1D). Gross evaluation of all AAAs shows biological variability in the morphologic characteristics and degrees of the AAAs in this model, with the largest cross-sectional AAA diameter ranging from 1.67 to 2.48 mm (Table 2). Comparison of in vivo and ex vivo measurements indicates the all AAAs were detected on sonography. Immunohistologic evaluation of elastin disruption and medial accumulation of macrophages confirmed established characteristics associated with this model of AAA development, as previously described (Figure 1E).¹⁸

Intramouse Variability of Short- Versus Long-Axis Measurements

The distribution of mean differences between baseline short- and long-axis measurements over resamples of data is illustrated in Figure 2A. Bland-Altman analyses of the agreement between short- and long-axis measurements of aortic diameter illustrate that the simple difference between measurements is not dependent on the mean, magnitude of difference, or starting point (Figure 2B). This finding is valid for overall comparison of short- and long-axis measurements as well as for specific aortic regions: infrarenal, suprarenal, and thoracic. The locally weighted scatterplot-smoothing trend line shows that discrepancies between short- and long-axis measurements do not vary with respect to the target measurement (Figure 2B). This suggests that the mean absolute difference is an adequate measure of discrepancy and a good summary of error between measurements. The associated 95%

confidence limits for mean absolute differences provide quantitative measures of precision between short- and long-axis measurements (Figure 2C). The overall difference between short- and long-axis measurements was 0.038 (95% confidence interval [CI], 0.031–0.046) mm; differences were 0.043 (95% CI, 0.029–0.058), 0.039 (95% CI, 0.027–0.052), and 0.033 (95% CI, 0.021–0.045) mm for infrarenal, suprarenal, and thoracic measurements of aortic diameters, respectively. These values show the reliability of in vivo sonographically derived parameters regardless of the imaging orientation or location along the target vessel.

In Vivo Versus Ex Vivo Diameters

The distribution of mean differences between baseline in vivo and ex vivo measurements is illustrated in Figure 3A. There was a trend in the Bland-Altman plots indicating a systematic bias between in vivo and ex vivo measurements of aortic diameter (Figure 3B). However, the systematic bias between measurements was independent of the mean, magnitude of difference, or starting point. In addition, the locally weighted scatterplot-smoothing analysis suggests that the discrepancy between pairs of in vivo and ex vivo measurements does not vary across the range of target measurements. The overall mean absolute difference between in vivo and ex vivo measurements (Figure 3C) was 0.085 (95% CI, 0.062–0.109) mm; differences were 0.037 (95% CI, 0.016–0.061), 0.119 (95% CI, 0.077–0.161), 0.147 (95% CI, 0.123–0.147), and 0.037 (95% CI, 0.019–0.059) mm for iliac, infrarenal, suprarenal, and thoracic measurements of aortic diameters, respectively (Figure 3C).

Intramouse Variability of Repeated Baseline Short-Axis Measurements of Aortic Diameter

The distribution of intramouse mean absolute differences in repeated baseline short-axis measurements is illustrated in Figure 4A. Evaluation of the agreement between repeated measurements illustrates that the simple difference between measurements is not dependent on the mean, magnitude of difference, or starting point (Figure 4B). Locally weighted scatterplot-smoothing evaluation shows no trend with respect to the target measurement. Corresponding estimates for mean absolute differences in repeated short-axis measurements with 95% confidence limits (Figure 4C) were 0.020 (95% CI, 0.012–0.028), 0.045 (95% CI, 0.033–0.057), 0.052 (95% CI, 0.037–0.067), 0.051 (95% CI, 0.034–0.073), and 0.037 (95% CI, 0.023–0.049) mm for iliac, infrarenal, right renal, suprarenal, and thoracic measurements of aortic diameters, respectively.

Intermouse Variability of Short- and Long-Axis Diameters

The distribution of mean differences in baseline short- and long-axis measurements across mice is illustrated in Figure 5, A and B, respectively. Analyses of intermouse agreement in short-axis (Figure 5C) and long-axis (Figure 5D) measurements illustrate excellent agreement independent of the mean, magnitude of difference, or starting point. A lack of a trend on locally weighted scatterplot-smoothing evaluation suggests that discrepancies do not vary with respect to target measurement. Estimated mean absolute differences with 95% CIs for short- and long-axis measurements across mice are illustrated in Figure 5, E and F. The estimated mean absolute differences between short-axis measurements across mice were 0.022 (95% CI, 0.019–0.024), 0.047 (95% CI, 0.042–0.053), 0.057 (95% CI, 0.052–0.062), 0.049 (95% CI, 0.044–0.055), and 0.039 (95% CI, 0.036–0.042) mm for iliac, infrarenal, right renal, suprarenal, and thoracic measurements of aortic diameters, respectively. Similarly, the estimates for long-axis measurements were 0.054 (95% CI, 0.044–0.064), 0.029 (95% CI, 0.024–0.034), and 0.046 (95% CI, 0.037–0.054) mm for infrarenal, suprarenal, and thoracic aortic diameters.

Discussion

In all instances of comparison, Bland-Altman evaluation suggests an agreement between measures without variation with the magnitude or anatomic region of the aorta. Furthermore, all accounts of nonparametric trend estimation showed symmetric variability around the locally weighted scatterplot-smoothing line, suggesting that mean absolute differences between measures do not vary with respect to target measurements, their range, or parts thereof. Overall, the quantitative estimates of precision are on the order of 0.1 to 0.01 mm in AAA and non-AAA regions regardless of variation in the sizes of the AAAs evaluated.

Studies of experimental AAAs, once confined to direct anatomic evaluation, are now aided by in vivo imaging. Yoshimura et al¹² used in vivo sonography to show regression of AAAs in mouse models by inhibiting c-Jun amino-terminal kinase (also known as stress-activated protein kinase). Using older-generation sonographic equipment, the images acquired in their study served the crucial purpose of delineating AAA regression in response to pharmacologic therapy. Although Satoh et al¹⁹ did not use in vivo imaging, their recent report on the role of cyclophilin A in the development of AngII-induced AAAs has rekindled the interest in AAA therapy.²⁰ It is reasonable to anticipate an increase in experimental strategies that incorporate in vivo imaging for longitudinal evaluation, eventually doing away with the need for mandatory ex vivo evaluation at specific time points. Given the relative ease of use, widespread availability, and reasonable startup costs, sonography is likely to take center stage as the modality of choice for contemporary in vivo imaging efforts targeted at AAA therapy.

We believe that the variability of measurements noted in this study largely stems from the variations in the measurement sites within regions of the aorta. It is difficult to posit that measurements are reproducibly achievable at the same exact spot, without error margins, during repeated in vivo evaluation in the same or different transducer orientations or between in vivo and ex vivo evaluations. Additional sources of variability include baseline respiratory motion, pulsation of the aorta, as well as operator dependence. The latter, arguably, was minimized in this study by the use of a single operator who acquired repeated measurements in different locations, at different times, and in a random order of animals. Regardless, our results suggest the fidelity of sonographically derived in vivo measurements and provide reassurance for their use in interrogating the aortas of living mice. Of note, there was a trend in the Bland-Altman plots indicating a systematic bias between in vivo (pre-euthanasia) and ex vivo (gross) measurements. This systematic bias appears largely in measurements acquired from the suprarenal and infrarenal regions, both prone to remodeling secondary to aneurysm formation (suprarenal), accelerated atherosclerosis due to AngII infusion, or both. The loss of friable pathologic aortic tissue from these regions during dissection and extraction contraction account for the systematic discrepancy between ex vivo and in vivo measurements of these regions. Consequently we were not interested in whether there was any general trend (slope = 0 versus not 0) but were interested in estimating the magnitude of any bias. In this context, despite the systematic bias precipitated by shrinkage, the Bland-Altman analyses show excellent agreement between in vivo and ex vivo measurements and suggest that simple differences between measurements are not dependent on the mean, magnitude of difference, or starting point. Furthermore, the discrepancies between pairs of in vivo and ex vivo measurements do not vary vis-à-vis the range of target measurement as estimated by nonparametric regression (locally weighted scatterplot smoothing).

The paramount importance of this study rests on the biological relevance of our precision estimates. Future studies of AAA regression may now use these estimates as guides for experimental designs. For example, a meaningful degree of regression (effect size) in

response to therapeutic intervention should be determined with reference to the confidence limits of the mean absolute difference for measurements of a target region. Similarly, in the event of repetitive surveillance, knowledge of these estimates is relevant in determining when a true change or difference is observed in the context of the experiment. We noted a substantial variation in the sizes of the aneurysms, ranging from 1.67 to 2.48 mm; however, the sonographic technique did well in these circumstances, with precision estimates that remained on the order of 0.01 mm regardless of AAA size. Similarly, analyses of measurements from nonaneurysmal aortas, including the iliac artery yielded similar results. The relevance of these findings may extend beyond studies of AAA to include in vivo imaging of atherosclerosis. The objective visualization of plaque and changes in its size will benefit from quantitative estimates of mean absolute differences in measurements at various locations. Furthermore, if plaque regression is the objective of an experiment, then the degree of regression sought should also be factored into the choice of the target plaque to allow for objective delineation of regression using sonography. Again, the knowledge of the variability estimates will help refine the design, planning, and execution of such experiments.

In conclusion, we have shown that the variability of measurements derived from sonography aneurysmal and nonaneurysmal aorta is in the order of 0.1 to 0.01 mm, with excellent agreement between measurements regardless of the size or region of the aorta evaluated. This study shows the reliability of in vivo sonography and provides quantitative estimates that could serve as references for future studies of AAAs and atherosclerosis, which may adopt in vivo sonography for longitudinal evaluation of therapeutic regression without mandatory euthanasia and assays of animals. In this context, the precision estimates reported in this study are indispensable in the design and execution of such studies in which objective determination of true regression is desired.

Notably, as the imaging frequency increases, the resolution also increases; thus, high-frequency ultrasound systems have superb resolution, but the trade off is that penetration decreases with increasing frequency. However, penetration was not a limitation in this study because microsonography has been proven sufficient for imaging larger small animals such as rats, rabbits, and other animals of comparable size.^{21,22}

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Abbreviations

AAA	abdominal aortic aneurysm
AngII	angiotensin II
Apo	apolipoprotein

CI confidence interval

Figure 1.

In vivo and ex vivo characterization of aneurysmal and non-aneurysmal aortas. **A**, Spectral Doppler signals of the aorta of a living mouse showing distinct spectral pattern of the aorta in comparison with the vena cava and a nonvascular focus. **B**, Representative baseline short-axis sonograms of a mouse aorta delineating the aorta at the iliac (postiliac bifurcation), infrarenal, right renal (takeoff of the right renal artery), suprarenal, and thoracic sites. **C**, Corresponding long-axis views of infrarenal, suprarenal, and thoracic aortas. **D**, Representative short- and long-axis images of a mouse aorta after angiotensin infusion delineating an AAA with a corresponding gross anatomic image. **E**, Representative histologic sections of an AAA showing elastin disruption (left), smooth muscle actin (middle), and macrophage infiltration (right).

Figure 2.

Intramouse variability: baseline short- versus long-axis measurements. **A**, Resampling distribution of mean absolute differences between baseline short- and long-axis measurements of aortic diameter. **B**, Bland-Altman plots of the agreement between short- and long-axis measurements of aortic diameter illustrating that the simple difference between measurements is not dependent on the mean, magnitude of difference, or starting point. This finding is valid for overall comparison of short- and long-axis measurements as well as for the specific anatomic sites evaluated: infrarenal, suprarenal, and thoracic. Locally weighted scatterplot-smoothing trend assessment shows that measurement discrepancies do not vary with respect to the target measurement. **C**, Estimates of mean absolute differences between short- and long-axis measurements with 95% confidence intervals. Faint background marks (+ ... +) represent observed absolute differences (raw data).

Figure 3.

Variability between pre-euthanasia (in vivo) and post-euthanasia (ex vivo) measurements. **A**, Resampling distribution of mean absolute differences between pre-euthanasia (in vivo) and ex vivo measurements of aortic diameter. **B**, Bland-Altman analysis of the agreement between in vivo and ex vivo measurements of aortic diameter indicating a systematic bias between measurements. However, excellent agreement is inferred because all values lie within 95% confidence limits. Notably, the bias is independent of the mean or magnitude of measures. Nonparametric regression (locally weighted scatterplot-smoothing) analysis shows no systematic variation in the discrepancies between pairs of measurements with respect to the range of target measurements. **C**, Estimated mean absolute differences between in vivo and ex vivo measurements with 95% bootstrap confidence limits. Faint background marks (+ ... +) represent observed absolute differences (raw data).

Figure 4.

Intramouse variability: repeated in vivo short-axis measurements. Short-axis images were acquired on each mouse 3 times over 3 days (once per day; see data in Table 1); diameters were determined at 5 anatomic locations. **A**, Resampling distribution of mean absolute differences between intramouse repeated in vivo short-axis measurements of aortic diameter. **B**, Bland-Altman analysis of the agreement between repeated measurements of aortic diameter illustrating that the simple difference between measurements is not dependent on the mean, magnitude of difference, or starting point. Locally weighted scatterplot-smoothing evaluation shows the lack of a trend in measurement discrepancies. **C**, Estimated mean absolute differences between repeated measurements with 95% bootstrap confidence intervals. Faint background marks (+ ... +) represent observed absolute differences (raw data).

Figure 5.

Intermouse variability: comparison across mice of short- and long-axis in vivo measurements. **A** and **B**, Resampling distribution of mean absolute differences between intermouse in vivo short-axis (**A**) and long-axis (**B**) measurements of aortic diameter. **C** and **D**, Bland-Altman analyses of intermouse agreement in short-axis (**C**) and long-axis (**D**) measurements of aortic diameter illustrating excellent agreement independent of the mean, magnitude of difference, or starting point. Locally weighted scatterplot-smoothing

evaluation shows no systematic trend in discrepancies with respect to the target measurement **E** and **F**, Mean absolute differences in short-axis (**E**) and long-axis (**F**) measurements across mice at specific anatomic sites with associated 95% bootstrap confidence limits. Faint background marks (+ ... +) represent observed absolute differences (raw data).

Table 1
Baseline Short- and Long-Axis Measurements of Aortic Diameter

Mouse	Short-Axis Diameter, mm				Long-Axis Diameter, mm			
	Iliac	Infrarenal	Right Renal	Suprarenal	Thoracic	Infrarenal	Suprarenal	Thoracic
01_B1	0.53	0.84	1.09	1.33	1.17	NR	NR	NR
01_B2	0.59	0.88	1.11	1.41	1.11	NR	NR	NR
01_B3	0.59	0.80	1.10	1.35	1.11	0.76	1.35	1.11
02_B1	0.57	0.85	1.21	1.23	1.19	NR	NR	NR
02_B2	0.55	0.84	1.09	1.35	1.11	NR	NR	NR
02_B3	0.61	0.88	1.09	1.33	1.21	0.80	1.35	1.17
03_B1	0.56	0.86	1.19	1.27	1.19	NR	NR	NR
03_B2	0.55	0.90	1.09	1.33	1.19	NR	NR	NR
03_B3	0.59	0.85	1.13	1.33	1.19	0.86	1.36	1.19
04_B1	0.53	0.70	1.23	1.35	1.21	NR	NR	NR
04_B2	0.57	0.80	1.10	1.39	1.13	NR	NR	NR
04_B3	0.55	0.80	1.11	1.35	1.13	0.80	1.37	1.23
05_B1	0.57	0.86	1.19	1.33	1.13	NR	NR	NR
05_B2	0.57	0.78	1.07	1.35	1.21	NR	NR	NR
05_B3	0.59	0.82	1.07	1.31	1.15	0.82	1.32	1.21
06_B1	0.55	0.84	1.09	1.29	1.19	NR	NR	NR
06_B2	0.57	0.84	1.04	1.32	1.17	NR	NR	NR
06_B3	0.57	0.86	1.09	1.37	1.17	0.87	1.32	1.16
07_B1	0.57	0.88	1.10	1.31	1.23	NR	NR	NR
07_B2	0.57	0.86	1.07	1.38	1.17	NR	NR	NR
07_B3	0.57	0.82	1.06	1.35	1.15	0.77	1.34	1.23
08_B1	0.61	0.84	1.13	1.33	1.14	NR	NR	NR
08_B2	0.59	0.88	1.07	1.31	1.21	NR	NR	NR
08_B3	0.59	0.76	1.09	1.33	1.19	0.87	1.30	1.15
09_B1	0.55	0.88	1.21	1.35	1.13	NR	NR	NR
09_B2	0.59	0.88	1.17	1.41	1.13	NR	NR	NR
09_B3	0.57	0.82	1.15	1.35	1.17	0.88	1.30	1.15

Mouse	Short-Axis Diameter, mm				Long-Axis Diameter, mm		
	Iliac	Infrarenal	Right Renal	Suprarenal	Thoracic	Infrarenal	Suprarenal
10_B1	0.57	0.86	1.15	1.23	1.17	NR	NR
10_B2	0.57	0.80	1.19	1.43	1.15	NR	NR
10_B3	0.57	0.80	1.11	1.37	1.17	0.78	1.33

Mouse measurements (mice 01–10) were determined at specific anatomic sites. Three baseline short-axis images (B1–B3) were acquired on 3 different days, 1 per day; long-axis images were acquired only on day 3. NR indicates not recorded.

Table 2
Aortic Diameters Before (In Vivo) and After (Ex Vivo) Euthanasia

Mouse	Diameter, mm			
	Iliac	Infrarenal	Suprarenal	Thoracic
02				
In vivo	0.59	1.02	2.40	1.37
Ex vivo	0.59	1.04	2.29	1.32
03				
In vivo	0.63	1.02	1.89	1.35
Ex vivo	0.64	0.85	1.72	1.37
04				
In vivo	0.59	0.92	2.46	1.31
Ex vivo	0.56	0.81	2.32	1.32
06				
In vivo	0.59	1.00	2.48	1.34
Ex vivo	0.50	0.78	2.22	1.27
07				
In vivo	0.57	0.96	2.38	1.39
Ex vivo	0.55	0.86	2.26	1.31
08				
In vivo	0.63	0.96	1.88	1.35
Ex vivo	0.60	0.87	1.67	1.36
09				
In vivo	0.63	1.02	1.88	1.35
Ex vivo	0.55	0.90	1.77	1.36

In vivo diameters were determined offline from acquired short-axis sonograms; ex vivo diameters were measured with digital calipers under microscopic guidance.