Dear Dr. Nguyen:

Please find attached the revised version of our manuscript titled “Measuring ascending aortic stiffness *in vivo* in mice using ultrasound.” We would like to thank the reviewers for their time and feedback which we found to be thoughtful and insightful. We have revised our manuscript according to their comments and added content in the introduction and discussion sections as suggested to improve clarity. We believe the manuscript to be greatly improved as a result.

Specifically, we included background information on the mechanical properties of the aorta. We also increased the working concentration of the drugs to reduce total volume infused and changed the infusion rates and figures to reflect the new working concentrations. We focused the data analysis section on compliance and removed the elastic modulus calculations due to concerns about the inaccuracy of the wall thickness measurements. We included additional figures (Figures 2 and 6) to improve the accessibility of this method. The figures and numbering have changed as a result. Finally, we expanded our discussion of the limitations of this method. Our response and the corresponding change to the manuscript are below each reviewer’s comment in italics. We look forward to publishing this manuscript with the *Journal of Visualized Experiments*.

Sincerely,

Maggie Kuo

Dan Berkowitz

Lakshmi Santhanam

**Reviewers' comments:**

Reviewer #1:

Manuscript Summary:

In this article, an experimental protocol to measure the pressure diameter relation of the ascending aorta of mice based on ultrasound imaging and invasive pressure measurements is described. Vascular biomechanical properties are an important factor in the pathophysiology of the cardiovascular system. These material properties of the arterial wall are not easily identified in vivo because not all neccessary factors can be measured. Therefore different indices have been developed which describe the structural stiffness of arteries based on clinically available measurements. The authors dexcribe a measurement protocol in which blood pressure is raised and lowered by two drugs to measure the diameter change over a wide range of blood pressure.

The pressure diameter relation is used to calculate several indices of (local) arterial rigidity.

Major Concerns:

The presented approach adequately describes the measurement of the diameter change over a wide range of blood pressure. The experimental protocol for conducting the measurements on the animals is well described. Important mechanical background information is not clearly presented or ommitted. The presented evaluation of the measured data in part is inadequate.

1) The limitations of the proposed method are not fully discussed, however: the accuracy of the ultrasound based diameter measurements are not given (resolution of the ultrasound images). This is especially important for the determination of wall thickness. The authors identify the artifacts introduced by rigid body movement of the ascending aorta as another source of uncertainty. This close to the heart this is especially pronounced and the measurements should be done distal to the aortic arch instead.

*We agree entirely that the main limitation of this method is the measurement uncertainty introduced by the movement of the ascending aorta, particularly given its proximity to the heart.*

*Stiffness measurement could be performed on the abdominal aorta because it can readily visualized by ultrasound. The thoracic aorta would be more difficult due to the rib cage. However, the ascending aorta is preferable for a number of reasons. First, it is easily identifiable by its proximity to the heart, which affords methodological benefits including ease of experimentation and measurement of stiffness in the same location between different animals. Second, ascending aorta stiffness has direct implications on cardiac function and coronary circulation, making these measurements clinically relevant. We have interest in investigating coronary function and developed this method in that context. Finally, ascending aorta stiffness is measured for many of the aortic stiffness studies in patient populations. Therefore experiments in animal models using this method could be directly compared to epidemiological findings.*

*We have now included an example of a pressure-diameter plot and corresponding compliance-mean aortic pressure plot in the Results section (Figure 6) to better illustrate the data spread. We have also included the standard deviation between diameter measurements (line 346). We have expanded our discussion of the limitations and approaches to addressing them (lines 430-452).*

2) The basics of vascular mechanics are not adequately presented, but are needed to understand the posibilities and limitations of this approach: elastic properties of the aortic wall (nonlinear elasticity, anisotropy); the different indices used to describe material (elastic modulus) and structural (compliance) stiffness.

*We have substantially revised the introduction to include background on vascular mechanics. We have included descriptions of the nonlinear elastic and anisotropic properties of the aortic wall (lines 104-128), as well as a comparison between elastic modulus and compliance (lines 138-141).*

3) The analysis of the measured data is confusing: (arterial) Compliance usually is calculated from the diameter difference (Dsys-Ddia) and the pulse pressure (Psys-Pdia). This compliance might be plotted versus the mean arterial pressure (MAP) to give a compliance / pressure plot which should show a nonlinear dependency of compliance on blood pressure (because of nonlinear elastic behaviour of the aortic wall). Therefore, using a linear fit on these data is not adequate.

*We have revised our data analysis and reevaluated our data in terms of this definition of arterial compliance (dD / dP) to minimize confusion (lines 350-357). We present plots of diameter as it changes with pressure and compliance as it changes with mean aortic pressure in the Results section (Figure 6). The compliance plot confirms the pressure-dependency of stiffness.*

*We derived the equation of C = d(Diameter)2 / dP from the definition of compliance C = (change in volume) / (change in pressure). Previously, we had analyzed our data by this definition of compliance and used a linear fit because this approach is done to calculate ex vivo stiffness by pressure myography. We saw our ultrasound method as an in vivo version of the pressure myograph method.*

4) The calculation of an elastic modulus is based on the (inaccurate) measurement of wall thickness and on simplifying assumptions concerning the boundary conditions of the aorta. It does not add new information over the calculation of compliance and should thus be avoided.

*We appreciate this concern, which was also echoed by Reviewer 3. For the data we had included, we excised the ascending aorta and measured the thickness by microscopy. We used one thickness value in our calculations based on the assumption that wall thickness does not change appreciably with pressure. We included elastic modulus because we thought that one of the novel aspects of this method compared to the invasive PWV approach is the ability to measure stiffness indices used to characterize mechanical properties of materials. Elastic modulus calculation was intended to illustrate how the pressure-diameter data collected can be used to calculate other stiffness indices besides compliance.*

*We have removed the elastic modulus calculations from the Results section because of this wall thickness measurement concerns. In the Discussion section, we included suggestions on how to obtain wall thickness measurements to calculate elastic modulus should an investigator be interested or better imaging technologies or techniques come along that make accurate in vivo thickness measurements feasible (lines 472-477).*

Minor Concerns:

li 130: "However, because large arteries are viscoelastic, PWV varies with arterial pressure." This variation is due to the non-linear elastic properties of the arterial wall and not the viscoelastic properties.

*We thank the reviewer for this important and insightful comment. We have incorporated this. (line 165).*

li 140-144: The indices presented in this approach (compliance) are indicative of the structural stiffness (not material). A major difference to PWV measurements is that PWV is averaged over the length of the aorta whereas the pressure / diameter measurement allows for locally varying measurements.

*We have incorporated this distinction between material and structural stiffness (lines 138-141). We have also included the distinction between stiffness measured by PWV and stiffness measured by this method (lines 162-164 and lines 179-180).*

li 146-149: PWV measurements usually rely on the "foot to foot" distance (phase shift of the end-diastolic time point of the pressure curve). This is not influenced by wave reflection.

*We have reread our original explanation and agree that an occlusion would alter the pressure wave form but not the time at which it arrives. We have removed this section.*

li 302 / 306: The adequate definition for arterial compliance for the measured data should use the diameter change over pressure change (C=ΔD/ΔP).

*We have reinterpreted our data according to this definition (lines 131 and 352).*

li 332 / Fig. 1: Please mention the ultrasound frequency used in the measurements. Add a scale bar to the image.

*Ultrasound probe frequency has been added (line 367 and 375). The entire recorded image from the ultrasound mainframe has been included to improve clarity. A scale bar is on these images (Figures 1, 2, and 3).*

li 336 / Fig. 2: Frequency of image acquisition (frames / s) should be mentioned.

*Acquisition sweep speed information has been included (line 375).*

Fig. 4: Add a title to the y-axis.

*We have improved our plots and labeled the axes more explicitly (Figure 4 and 5).*

li 354 / Fig. 6: Replace figure (cf. 3 above).

*We have removed the elastic modulus data as suggested.*

li412-416: This approach relies on using vasoconstrictors or vasodilators to (indirectly) modulate blood pressure. Changing the tone of vascular smooth muscle cells (VSMC) will significantly change the stiffness, thus limiting the use of this technique to elastic arteries. Even in elastic arteries modification of VSMC will change wall stiffness to some extent.

*This is a possible limitation to this protocol. However, a study by our collaborator, A. Avolio, showed that PWV measured by changing blood pressure pharmacologically or by venous return was not different. Their findings demonstrate that the vasoactive effects of the drugs are in the resistant arteries. We have included this in our discussion (line 454-459).*

Reviewer #2:

Manuscript Summary:

This study describes a methodology for assessing aortic stiffness in mice in vivo using ultrasound. Clearly, if validated, such a technique could be useful in studying aortic pulse wave velocity in mouse models in vivo. However, it still involves anaesthetising the animals and invasive insertion of a catheter into the aorta.

1. How does the aortic pulse wave velocity, measured using this technique, equate to aortic pulse wave velocity measured using the transit time methodology?

*This method measured stiffness from the aorta’s pressure-dimension relationship and does not measure aortic pulse wave velocity (PWV).*

2. A recent study (Leloup et al, Hypertension 2014;64:ePub) has used applanation tonometry to assess pulse velocity in mice. The authors should compare and contrast their technique with that of the Leloup group.

*The data collected by the tonometry technique described by Leloup et al. is the same as the data collected by non-invasively measuring PWV. The main limitation of measuring PWV non-invasively, whether by Doppler ultrasound or applanantion tonometry, is that the measurement yields a single point value of stiffness. Because PWV is influenced by blood pressure, single point PWV measurements must be normalized to mean arterial pressure to make meaningful comparisons between experimental groups.*

*We have now referenced this study in this manuscript and expanded our explanation on the limitations of single point measurements of PWV in the introduction (lines 160-168).*

Reviewer #3:

Manuscript Summary:

This study describes a protocol by which to measure aortic stiffness in vivo that is an alternative to pulse wave velocity, which is plagued by dependence on blood pressure and heart rate. The authors should be commended for a nice study and should consider the following comments:

Major Concerns:

1. One of the major confounds of measuring vascular stiffness other than by pulse wave velocity is that many of the equations, including elastic modulus, require some input of wall thickness, which is difficult to accurately obtain by echo. The resolution of the Vevo2100 is around 25 um (1 pixel is about 25 um square), and a typical mouse aorta has a wall thickness of around 50-75 um, so measuring wall thickness in these mice at only 2-3 fold higher than the resolution seems like it would introduce a good bit of variability into the data. The authors should check the accuracy of their Vevo wall thickness measurements by collecting aortas after ultrasound measurements to determine how the aortic morphometrics compare with those measured in vivo (may require vessel myograph to pressurize vessel to similar extent as in vivo).

*This is an important concern and we agree that wall thickness is difficult to measure accurately using ultrasound. Please also see our response to Reviewer 1, #4. We removed the elastic modulus calculation and focused on compliance calculation instead (lines 350 – 357 and Figure 6).*

2. It may be useful to also compare elastic modulus with another clinically-used index, beta stiffness, which can be calculated from diameter and pressure measurements. Moreover, how to the elastic modulus measurements compare with those obtained by pulse wave velocity?

*We have removed the elastic modulus calculation and focused the analysis on the calculation of arterial compliance.*

*Stiffness measured by this technique is different than PWV in that PWV is stiffness averaged over a length of aorta while this technique is local stiffness. We have made this distinction in the introduction (line 164 and lines 179-182)*

3. When in vivo infusions are performed, it is best to keep the total infusion volume at/below 10% of the estimated total blood volume (at/below about 200 uL in the normal mouse) to avoid activating baroreceptor reflex systems controlling blood pressure. The authors infused about 660 uL total into each mouse, which is well above that required to elicit a central response. Admittedly, the doses of drugs infuse apparently were sufficient to overcome any central responses as evidenced by the blood pressure data. Nonetheless, there should be some effort to increase the stock concentration of Phe and SNP so that less volume can be infused into each mouse. This may also affect the BP stabilization time for each dose infused. Finally, it's best to represent the Phe and SNP doses per kg body weight (e.g. ug/kg/min).

*This is an important methodological consideration, and we thank the reviewer for bringing this to our attention. We have changed the stock concentrations and infusion rates to decrease the amount of total volume infused (lines 290 – 318). Total volume infused with this new protocol is ~100 µL. Dosing units have also been changed to µg/kg/min.*

4. Protocol questions/concerns:

-1.2: Do you heparinize the saline for venous cannulation?

*Heparin saline is used to prevent potential clotting in the cannula during the catheter insertion procedure, but PE and SNP are prepared in saline alone. We have included this in the protocol (line 203-204 and line 210-211).*

-3.4: What size suture is used to secure the catheter?

*We use braided silk 6-0 suture. We find that silk is easiest to knot securely. The materials and vendors we use are in the materials list.*

-3.6: In this reviewer's experience, inserting a 1.2F pressure-tip catheter into the mouse femoral artery almost always requires the topical administration of a vasodilator such as lidocaine to make the artery sufficiently large enough to accommodate the catheter. Did the authors find this necessary? If not, the authors may consider mentioning the potential use of vasodilators to aid in catherterization. I also find that bending the bevel of a 30-Ga needle (using fine needle drivers) about 60 degrees makes a nice introducer for cannulation. Also, it is unclear how far the catheter is advanced up the aorta? Given that the mechanical measurements are made at the ascending aorta, it would be best to measure pressure closest to that point, but the pressure drop down the aorta is likely negligible. In any case, some clarification of catheter position should be provided.

*We have incorporated your techniques as suggestions to facilitate femoral cannulations for other investigators (lines 416-417).*

*We find that lidocaine is not necessary to insert the catheter. Our experience may be because the insertion point is done quite proximally along the femoral artery. Also, the mice are anesthetized at 2% isoflurane, which has vasorelaxing properties that may also aid in the cannulation procedure. We also find that opening the membrane sheath surrounding the femoral artery-vein bundle dramatically increases the femoral artery diameter.*

*In general, we advance it into the abdominal aorta to leave the thoracic region clear for imaging. However, as you mention, since pressure difference along the aorta is not significant, we do not find it necessary to be exacting in the catheter placement location. We have included this clarification into the protocol (line 270) and discussion (line 420-428).*

-Some commentary about catheter setup and calibration would be useful.

*We have included catheter setup and calibration in the protocol (lines 240-242 and 263-265).*

Minor Concerns:

1. Are the blood pressure values reported as mean arterial pressure? Please clarify in Figure 2, and provide y-axis scale bar on Figure 4.

*We have labeled the pressure measurement more precisely as “mean aortic pressure’ or “absolute aortic pressure” (Figure 5 and Figure 6). Y-scale axis bar has been included in the blood pressure traces (Figure 4.)*

2. Methods 1.4: Saline solution is not an ointment for eyes. Do you use either ointment or saline?

*We do not find it necessary to add saline solution or vet ointment. Their use was included in the protocol to follow the format of this journal.*