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TITLE:

Quantification of Atherosclerosis in Mice

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SUMMARY:

Murine models of atherosclerosis are useful tools to investigate pathogenic pathways on a molecular level, but require standardized quantification of lesion development. This protocol describes an optimized method to determine lesion size in the major arterial vessels including the aortic root, aortic arch, and brachiocephalic artery.

ABSTRACT:

Cardiovascular disease is the main cause of death in the world. The underlying cause in most cases is atherosclerosis, which is in part a chronic inflammatory disease. Experimental atherosclerosis studies have elucidated the role of cholesterol and inflammation in the disease process. This has led to successful clinical trials with pharmaceutical agents that reduce clinical manifestations of atherosclerosis. Careful and well-controlled experiments in mouse models of the disease could further elucidate the pathogenesis of the disease, which is not fully understood. Standardized lesion analysis is important to reduce experimental variability and increase reproducibility. Determining lesion size in aortic root, aortic arch, and brachiocephalic artery are common endpoints in experimental atherosclerosis. This protocol provides a technical description for evaluation of atherosclerosis at all these sites in a single mouse. The protocol is particularly useful when material is limited, as is frequently the case when genetically modified animals are being characterized.

INTRODUCTION:

Cardiovascular disease is the main cause of death in the world with ischemic heart disease and stroke accounting for one in every four deaths[1]. Most cases are caused by atherosclerosis, a disease characterized by a slow build-up of lipid-laden plaques with signs of chronic inflammation in large- and medium-sized arteries[2]. The disease usually remains unnoticed over several decades until a rupture or erosion of the plaque elicits an arterial thrombosis that leads to ischemic tissue damage.

A normal artery consists of an intima layer with endothelial cells and sparsely distributed smooth muscle cells, a media layer with smooth muscle cells and elastic lamellae, and a surrounding adventitial layer with loose connective tissue[3]. An intimal retention of LDL offsets atherosclerosis development[4]. Accumulation and modification of lipoproteins lead to aggregation and entrapment within the arterial intima[5]. An inflammatory response is evoked by the trapped and modified lipoproteins[6]. Endothelial cells start to express adhesion molecules, such as VCAM-1 at sites in the arterial tree with turbulent blood flow, leading to recruitment of circulating monocytes and other leukocytes[7]. The infiltrating monocytes differentiate into macrophages that engulf lipid with ensuing transformation to macrophage foam cells[8].

Atherosclerosis has been studied in mouse models with increasing frequency since the mid-1980s. C57BL/6 is the most commonly used inbred mouse strain for these studies, and it is used as the genetic background for the majority of genetically modified strains[9]. This strain was established in the 1920's[10], and its genome was published in 2002[11]. Experiments in mouse models have several benefits: the colonies reproduce fast, housing is space-efficient, and inbreeding reduces experimental variability. The model also allows for genetic manipulations, such as targeted gene deletions and insertion of transgenes. This has led to new pathophysiological understanding of the disease and new therapy targets[12].

Wild-type C57BL/6 mice are naturally resistant to atherosclerosis. They have most of the circulating cholesterol in HDL, and complex atherosclerotic lesions are not formed even when fed a high-fat and high-cholesterol diet[13]. Hypercholesterolemic mice, such as *Apoe*$^{-/-}$ on the C57BL/6-background, are therefore used as experimental models of atherosclerosis[14,15]. The lack of ApoE impairs hepatic uptake of remnant lipoproteins and severely perturbs lipid metabolism. In *Apoe*$^{-/-}$ mice, circulating cholesterol is predominantly in VLDL particles, and the mice develop complex atherosclerotic plaques on a regular chow diet.

Ldlr$^{-/-}$ mice mimic the development of atherosclerosis seen in humans with familial hypercholesterolemia[16]. The *Ldlr*$^{-/-}$ mice need a Western type diet to develop atherosclerosis[17]. Western diet mimics human food intake and usually contains 0.15% cholesterol. The LDL receptor recognizes ApoB100 and ApoE and mediates uptake of LDL particles through endocytosis. LDL receptors are fundamental for liver clearance of LDL from circulation, while LDL receptor expression in hematopoietic cells does not influence this process. This opens the possibility for bone marrow transplantation of *Ldlr*$^{+/+}$ cells into hypercholesterolemic *Ldlr*$^{-/-}$ recipients and assessment of atherosclerosis development. Bone marrow chimeras have commonly been used to study the participation of hematopoietic cells in experimental atherosclerosis. However, bone marrow transplantation could influence the size and

composition of atherosclerotic plaques, making interpretation of results ambiguous.

Different variants of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice with additional genetic alterations have been developed to study specific processes of the disease[18]. One example is human *APOB100*-transgenic *Ldlr*$^{-/-}$ (*HuBL*) mice that carry the full-length human *APOB100* gene[19,20]. These mice develop hypercholesterolemia and atherosclerosis on a regular chow diet. However, the development of complex atherosclerotic plaques takes at least six months and shorter experimental protocols usually use Western diet[21]. A large fraction of plasma cholesterol is circulating in LDL particles, which gives *HuBL* mice a more human-like dyslipidemic lipoprotein profile compared to *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice. *HuBL* mice also allow studies of human apoB as an autoantigen[22].

The mouse models of atherosclerosis develop complex atherosclerotic plaques with shared features of human disease. However, the plaques are fairly resistant to rupture with ensuing myocardial infarction. Atherothrombosis is only sporadically detected and experimentally challenging to assess[23-25]. Special models of plaque rupture have been developed, but the experimental field lacks a reliable and reproducible model for assessment of plaque stabilizing agents.

Quantification of atherosclerosis has been reported in numerous ways in the literature. Recent efforts have tried to standardize experimental design, execution, and reporting of animal studies[26]. Investigators have different preferences and techniques adapted to their laboratories. Most research projects are also unique in a way that they require some protocol modifications. Due to the multifactorial nature of the disease, optimal controls vary between projects. Local conditions and lack of standardization may cause observed differences in disease development, which hampers advances of the research field. Differences in experimental variability also means that statistical power calculations need to be based on pilot studies under local conditions.

Quantification of atherosclerosis is recommended at several locations in the vascular tree. This protocol describes how to obtain results from the aortic root, the aortic arch, and the brachiocephalic artery in a single mouse, in addition to leaving the rest of the thoracoabdominal aorta for other analyses. En face preparations allow rapid quantification of lipid-laden plaques in the aortic arch. Disease burden in the brachiocephalic artery can also be quantified if the specimens are carefully displayed. The more time consuming cross-sectioning of the aortic root leaves several sections available for detailed evaluation of plaque composition.

PROTOCOL:

All animal experiments require approval by ethical authorities.

1. Mouse sacrifice and microdissection of aorta

1.1. Sacrifice the mouse by CO_2 asphyxiation and record weight.

1.2. Spray the mouse with 70% ethanol to avoid fur contamination of the samples. Place the mouse in a supine position. From the jugular notch, make a midline incision using Mayo scissors extending it almost down to the pubic bone.

CAUTION: High percentage ethanol is highly flammable and could cause serious eye irritation. Take precautionary measures.

1.3. Use a 23 gauge needle to exsanguinate the mouse by cardiac puncture through the thorax wall. This procedure usually yields 750 µL of blood from a 20 week old mouse. Typically, collect half of the volume in a tri-potassium EDTA-coated tube and the other half in a serum or lithium heparin-coated tube. Gently turn the tubes and keep them at room temperature until further processing.

1.4. Use Mayo scissors to cut the parietal peritoneum in the midline to open the abdominal cavity. Hold the xiphoid process with tissue forceps and cut open the peritoneum laterally on both sides and continue to open the diaphragm.

1.4.1. Use the Mayo scissors to open the chest cavity by cutting through the rib cage as laterally as possible. This will enable wide angles for the instruments while microdissecting the aorta later on.

1.5. Make an incision in the right auricle for perfusion fluid drainage. Insert a 27 gauge needle through the apex of the heart in cranial direction. Keep the needle fixed in the left ventricle while slowly perfusing the mouse with 10 mL ice-cold phosphate-buffered saline (PBS) during minimum 2 minutes. Observe the liver shifting in color and getting paler.

NOTE: Some protocols use paraformaldehyde perfusion, but this interferes with several downstream applications, such as immunohistochemistry analysis of lymphocytes. Therefore, no perfusion fixation with paraformaldehyde is performed in this protocol.

1.6. Dissect organs of interest (e.g. lymph nodes, spleen, liver, intestine, inguinal fat pads, kidneys, etc.) using anatomical forceps and dissecting scissors.

1.7. Cut trachea and esophagus on the right side of the heart without damaging the aortic arch. Cut the diaphragm and structures attaching the viscera to retroperitoneum, leaving the heart, aorta, and kidneys in situ. Fold away the lungs and viscera caudally and cover them with a napkin to begin retroperitoneal microdissection of para-aortic lymph nodes and abdominal aorta.

1.8. Start microdissection under a stereomicroscope at 6x magnification. Begin to dissect the aortic bifurcation by lifting surrounding tissue with Dumont forceps and cutting under tension with Vannas scissors.

1.8.1. Continue dissection of the abdominal aorta cranially. Cut abdominal branches from the aorta and free the aorta proximally through the aortic hiatus in the diaphragm.

NOTE: Microdissection requires accurate hand-eye coordination through the stereomicroscope, which takes some practice to master.

1.9. Remove the adipose tissue covering the thoracic aorta. Carefully dissect dorsally of the thymus to free the aortic arch with branches. Continue dissecting the carotid arteries as distally as possible in the thoracic cavity. In special cases, neck dissection could be performed to include the carotid bifurcation.

1.10. Clean the instruments by consecutive rinses in deionized water, RNase decontamination solution, 70% ethanol, and PBS before actually cutting the aorta. Lift the heart by the apex with the forceps. Cut the aorta close to the heart and place the whole heart in a tube with PBS. The heart could be stored on ice for a couple of hours before continued processing and cryomounting the aortic root.

1.11. Cut the aortic arch according to **Figure 1A**. Put the aortic arch in a tube containing 1 mL of 4% formaldehyde overnight at 4 °C. The specimen could be stored in this manner for several years before pinning and analysis.

CAUTION: Formaldehyde may cause cancer, allergic skin reactions, and is harmful if swallowed. Use personal protective equipment as required.

1.12. Dissect the remaining descending aorta and put it in an RNA stabilization solution or snap freeze it for subsequent RNA analysis or other application. Optimizing work flow to minimize dissection time is crucial to avoid excessive RNA degradation.

1.13. Put the blood collection tubes (collected in step 1.3) in a centrifuge. Spin down the separate plasma and serum tubes at 1,500 x *g* for 15 minutes at room temperature. Carefully transfer the plasma and serum to microcentrifuge tubes and store at -80 °C. Collecting both EDTA and heparinized plasma or serum leaves possibilities for multiple downstream applications.

1.14. Place the heart on a cork bed with the ventral side facing up. Fix the heart to the cork with a needle through the apex. Hold the base of the heart with anatomical forceps.

1.14.1. Use a scalpel to cut away the apical 2/3 of the heart with the direction of the cut being as a line between the two auricles with the scalpel angled 20° caudally in the sagittal plane and 20 degrees cranially in the transversal plane (**Figure 1B**).

1.15. Embed the aortic root in optimum cutting temperature (OCT) compound, which surrounds, but do not infiltrate the tissue. Immerse the base of the heart in OCT compound.

Gently squeeze the heart with the forceps to fill the aortic root with OCT and remove any air bubbles.

1.16. Transfer the specimen to the bottom of a cryomold filled with OCT. The aortic root should now be perpendicular to the bottom surface. Put the mounted heart on dry ice to freeze. Store the specimens in zip lock bags in -80 °C until pursuing cryosectioning according to section 3 in this protocol.

[Place **Figure 1** here]

2. En face analysis of aortic arch and brachiocephalic artery

2.1. Prepare pinning beds for en face analysis of aortic arches. Fold a segment of paraffin wax film eight times to make a flat 25 mm x 25 mm surface. Wrap it with black electric insulation tape to make a dark background for the aorta. Place a label on the backside of the pinning bed and use a lead pencil to write the mouse identification number (normal pen ink will disappear in the staining process).

2.2. Transfer the aortic arch to the pinning bed and place a drop of PBS on top of it. Begin cleaning the aorta from remaining periadventitial adipose tissue under a stereomicroscope.

2.2.1. Use Vannas scissors and Dumont forceps to gently peel away all surrounding adipose tissue without manipulating or damaging the aorta. The Sudan IV will stain adipose tissue brightly and it is crucial to remove all such tissue at this point.

NOTE: Keep the aorta moist at all times applying additional PBS when needed.

2.3. Cut open the aorta in the coronal plane by introducing the Vannas scissors in the aortic lumen to expose the intimal surface. Begin to cut the outer curvature of the ascending arch in distal direction and continue to cut open the branches including the brachiocephalic artery. Spare the dorsal part of the descending thoracic region.

2.3.1. Cut open the lesser curvature and fold open the aorta to display the intimal surface.

NOTE: This step requires fine motor skills and needs some practice to master.

2.4. Pin the open arch to the pinning bed using the blunt end of minutien insect pins. Use a micro Castroviejo needle holder to put the pins in place. Gently bend the pins away from the specimen when in place. Pin the aorta flat on the bed without stretching the specimen. Store the pinned arch facing downwards in a Petri dish filled in PBS at 4 °C.

NOTE: The protocol can be paused here.

2.5. Prepare a working solution of Sudan IV. Mix 1 g of Sudan IV powder, 100 mL of 70%

ethanol, and 100 mL of acetone in a dark bottle and gently stir for 10 minutes. There is no need to filter the solution and it can be used for a couple of months if kept dark at room temperature. If the staining color is not satisfactory, a new solution can be made and the specimens stained again.

CAUTION: Acetone is a flammable liquid that could cause serious eye irritation. Store in a well-ventilated place and take precautionary measures when handling.

2.6. Arrange five Petri dishes on the lab bench: one filled with 70% ethanol, one filled with Sudan IV working solution, two filled with 80% ethanol, and one filled with PBS.

2.6.1 Start with rinsing the specimen in 70% ethanol for 5 minutes by placing the pinning bed in the first Petri dish with the arch facing downwards. Transfer the specimen to the Sudan IV working solution and let it stain the arch for 7 minutes.

2.6.2 Next, rinse in 80% ethanol for 3 minutes twice to destain the normal intimal surface. Destaining time could be adjusted to optimize results. Lastly, rinse in PBS before putting the specimen back into the original Petri dish.

2.7. Acquire micrographs using a stereomicroscope at 10 times magnification connected to a digital camera. Take pictures of the pinned arch submerged in PBS using small metal weights (20 mm x 10 mm x 5 mm) to hold the pinning bed to the bottom of a Petri dish. Place a ruler next to the aorta for calibration of the image.

2.8. Use an image analysis software (e.g. ImageJ) to determine lesion area and total intimal surface. In lack of anatomical landmarks to define the aortic arch, measurement is usually performed from the start of the ascending aorta down to the first intercostal branch (**Figure 2A**). Use the area quantification feature in the software to manually encircle the total intimal arch area.

NOTE: The lesion quantification should be done in a blinded fashion and it is advisable that a second investigator confirms the results.

2.8.1 In ImageJ, select the polygon selection tool and encircle the total arch area by repetitive clicks. Then select measure in the analyze menu to display the total arch area in the result window.

2.8.2 Next, encircle all Sudan IV-stained plaques in the arch. Sudan IV is a lysochrome diazo dye that stains lipids, triglycerides, and lipoproteins with an orange-red color. In ImageJ, select the freehand selection tool and encircle all plaques while pressing the Alt key. Click measure in the analyze menu to display lesion-free arch area in the result window.

2.8.3 Calculate relative lesion area by subtracting the lesion-free area from the total arch area and then dividing the result with the total arch area.

2.9. Carefully pin the subclavian and carotid arteries to enable lesion quantification in the brachiocephalic artery (Figure 2A). Quantification of lesions in the subclavian arteries and the common carotid arteries is usually very challenging and not meaningful, respectively.

2.9.1 In ImageJ, encircle both pieces of the braciocephalic artery using the polygon selection tool while pressing the shift key. Click measure in the analyze menu to display the total brachiocephalic artery area in the result window.

2.9.2 Next, select the freehand selection tool and encircle all plaques in brachiocephalic artery while pressing the Alt key. Click measure in the analyze menu to display the lesion-free brachiocephalic artery area in the result window.

2.9.3 Calculate relative lesion area by subtracting the lesion-free area from the total brachiocephalic artery area and then dividing the result with the total brachiocephalic artery area.

3. Cryosectioning of the aortic root

3.1 Set the cryostat temperature at -20 °C and section thickness to 10 µm. Mount the OCT block containing the aortic root on the specimen holder with the ventricular tissue facing outward. While starting to cut, fine tune the alignment of the section surface to be parallel to the specimen holder.

3.2 Remove excessive surrounding OCT to make it easier to collect sections without folds. The aortic root should now be positioned perpendicular to the knife blade given that the base of the heart was placed correctly in the mold.

3.3 Collect initial control sections on ordinary microscope slides, which will be discarded. The first sections should only contain heart muscle tissue. Progress the sectioning by 200 µm at the time. Collect a section and check the progress with a light microscope.

3.4 When getting closer to the left ventricle outflow tract, check every 100 µm under the microscope. When initial indications of a vessel wall are observed, slow down the pace to 50 µm.

NOTE: When the first aortic cusp appears, this will be point zero for collecting sections. It can be difficult to see when the cusps appear exactly, but an exact localization is crucial to perform comparisons of lesions in the same region.

3.5. Tilt the specimen towards the point zero cusp to align the section plane with the two other cusps. This is crucial for obtaining true cross sections of the aorta. Make a drawing of the aortic root, indicating the cusps as they appear, and count every 10 µm section that are cut from point zero onwards.

3.5.1. When a second cusp appears, slightly tilt the specimen again away from the cusp to align the specimen with the third cusp. The level difference between the cusps should not exceed 50 µm. Start to collect sections on slides from level 90 µm and onwards.

3.5.2. Collect sections according to the slide planning in **Figure 3**. The collection of sections may be started from 190 µm if the aortic root is more than 50 µm tilted, to allow further space to align the root in a straight position. Continue sectioning until reaching level 800 µm from point zero. If there are still visible plaques at this level, the collection could be expanded to 1000 µm.

NOTE: A simplified slide organization is presented in Supplemental figure 1, which could increase sectioning speed. The optimal slide planning should be decided depending on the project plan.

3.6. Fix the sections after collection.

3.6.1. Fix the sections collected for Oil Red O staining and Picrosirius red staining of collagen in 4% formaldehyde for 10 minutes. Rinse in deionized water, dry, and store in room temperature until pursuing with section 4 in this protocol. If slides should be stained with Oil Red O right away and are still wet, place them in 60% isopropanol for 1 minute to speed up the drying process.

CAUTION: Isopropanol is a flammable liquid that could cause serious eye irritation and may cause drowsiness or dizziness. Store in a well-ventilated place and take precautionary measures when handling.

3.6.2. Fixate the sections collected for immunohistochemistry or immunofluorescence in ice cold pure acetone for 10 minutes. Dry in room temperature for 30 minutes. Store sections in -20 °C.

[Place **Figure 3** here]

4. Oil red O staining and quantification of atherosclerosis in aortic roots

4.1 Prepare a saturated Oil Red O solution by dissolving 1 g of Oil Red O in 100 mL of isopropanol. Stir the solution in a dark bottle for 1 hour at room temperature. The saturated solution can be kept for several months.

NOTE: It is advisable to have designated laboratory equipment for Oil Red O staining since it is difficult to clean equipment that has been in contact with the solution.

4.2 Prepare a working solution by mixing 75 mL of the saturated Oil Red O solution with 50 mL of deionized water. Let stand in room temperature for 10 minutes. Filter through a

qualitative filter paper.

4.3 Place the slides in Oil Red O working solution for 20 minutes. Rinse in tap water for 5 minutes.

4.4 To assist tissue visualization, stain with Mayer's hematoxylin for 1 minute. Rinse in lukewarm tap water for 5 minutes. All nuclei should now be stained in blue color, adjust staining time to optimize the result.

4.5 Mount slides in an aqueous mounting medium (e.g., Kaiser's glycerol gelatin). Warm Kaiser's glycerol gelatin to 40 °C to make it fluid before use. It is not necessary to dry the slides since the mounting medium is water based. Be careful to avoid air bubble formation when adding the cover glass.

CAUTION: Kaiser's glycerol gelatin contains phenol, which is suspected of causing genetic defects. Use personal protective equipment as required.

4.6 Acquire digital micrographs using a camera connected to a light microscope. Usually the full vessel wall and lesion boundaries could be clearly visualized by 50 times magnification. Save high resolution images, preferably in tagged image file format (TIFF).

4.7 Perform analysis of lesion size using a computer-assisted image analysis software system. Oil Red O is a lysochrome diazo dye that stains neutral lipids and visualizes atherosclerotic plaques with an intense red color, which assists lesion quantification.

NOTE: The lesion quantification should be done in a blinded fashion and it is advisable that a second investigator confirms the obtained results.

4.7.1 Use the area quantification feature in the image analysis software to define the total vessel area by encircling the external elastic lamina of the aortic vessel wall (**Figure 2B**). In ImageJ, select the polygon selection tool and encircle the area by repetitive clicks. Then select measure in the analyze menu. The total vessel area is displayed in the result window.

4.7.2 Continue to quantify the atherosclerotic lesions in the intimal layer of the vessel, defined by the internal elastic lamina and the luminal boundary. Usually lesions on valve cusps are excluded from the measurement[27]. In ImageJ, select the freehand selection tool and encircle all plaques while pressing the Alt key. Select measure in the analyze menu to display the lesion-free vessel area in the result window.

4.7.3 Calculate the relative lesion area by subtracting the lesion-free area from the total vessel area and then dividing the result with the total vessel area.

NOTE: Calibrate the results in the image analysis software according to the used magnification to obtain absolute lesion area in square micrometer.

4.8 Define the Oil Red O-stained area in the lesions by using a color threshold feature in the image analysis software to calculate the percentage of Oil Red O positive area of total lesion area.

4.8.1 In ImageJ, encircle all lesion area by using the freehand selection tool while pressing the shift key. Select measure in the analyze menu to display total lesion area is in the result window.

4.8.2 Select clear outside in the edit menu. Change the image type to 8-bit in the image menu.

4.8.3 Set a red threshold for Oil Red O negative area by selecting threshold in the adjust submenu of the image menu. Click apply. Make the image binary by selecting this option in the binary submenu of the process menu.

NOTE: Usually Oil Red O staining varies between batches. Hence, color thresholding is only recommended within the same staining batch. The result should be presented along with a description how the threshold was determined and standardized.

4.8.4 Analyze the picture by selecting analyze particles in the analyze menu and click ok. Total Oil Red O negative lesion area is now displayed in the summary window. Calculate the relative Oil Red O positive area by subtracting the Oil Red O negative area from the total lesion area and then dividing with the total lesion area.

REPRESENTATIVE RESULTS:

In mouse models of atherosclerosis the most prominent lesions tend to develop in the aortic root and aortic arch. This protocol describes quantification of atherosclerosis in the aortic root, the aortic arch, and the brachiocephalic artery in a single mouse. Measurable lesions in thoracic descending aorta and abdominal aorta are only present in animals with advance disease. In this protocol, these parts are not analyzed for atherosclerotic burden, but saved for subsequent analysis of mRNA levels or other analyses. Serial sections of atherosclerotic lesions in the aortic root is usually displayed in a graph with lesion size on the *y*-axis and distance to the aortic sinus on the *x*-axis[28]. True cross-sections are crucial for lesion size quantification. Oblique sections can overestimate lesion sizes and a tilting of only 20 degrees could overestimate the absolute lesion surface by 15%[29]. However, calculating the lesion fraction of total vessel area makes the result less sensitive to possible angle differences during sectioning (**Figure 4A**). An appropriate statistical method to detect differences between groups is usually a regular 2-way analysis of variance (ANOVA). Bonferroni post-tests are then carried out to detect differences at certain levels. Fisher's least significant difference could also be used as a follow-up test to ANOVA. It reduces the likelihood of type II statistical errors, but do not account for multiple comparisons. In addition, it could be illustrative to calculate area under the curve or the average lesion size per mouse and present the data in a dot plot to further visualize individual variation within the groups (**Figure 4B**).

Oil Red O is a fat-soluble bright red diazo dye, which stains neutral lipids. Polar lipids in cell membranes are not stained. Oil Red O staining can be performed on fresh, frozen, or formalin-fixed samples, but not on paraffin-embedded samples due to the removal of lipids in the required deparaffinization process. A quantification of lesional lipid accumulation could be performed by color thresholding the Oil Red O positive area of total lesion area (**Figure 4C**). Hematoxylin produces a blue staining of cell nuclei, which is helpful to visualize plaque morphology. The right and left coronary arteries usually diverge from the aorta around 250 µm from the aortic sinus[27], which often coincide with the most prominent lesion sizes. Cross-sections from this region is often displayed as representative results (**Figure 4D**).

[Place **Figure 4** here]

Oil Red O could be used for staining of en face prepared aortas, but this protocol uses Sudan IV, another convenient fat-soluble diazo dye. Sudan IV clearly visualizes atherosclerotic plaques in an orange-red color by staining lipids, triglycerides, and lipoproteins. Removing the dark background in representative images of the en face aortic arches could enhance the visual display (**Figure 5A**). Usually lesion size is normally distributed within groups, allowing statistical testing with Student's *t*-test between groups. A dot plot that shows both individual mice and the mean, which is compared between groups, is an informative way to display the results (**Figure 5B-C**). Since the variation within groups typically is different between locations in the vascular tree, separate power calculations are usually needed. Unnecessary variation can be avoided by method proficiency and protocol standardization. Obtaining statistically significant results is important, but the biological relevance for an observed difference always needs to be considered as well.

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1. Heart and aortic arch in situ. (**A**) Lungs, trachea, esophagus, and thymus are removed to display the aortic arch in situ in a 20 weeks old female *Apoe*$^{-/-}$ mouse on regular chow diet in a micrograph with a 2 mm scale bar. The dotted lines indicate where to cut the aortic arch and its branches. (**B**) A schematic depiction of the heart and aorta. The dotted line in red indicates where to cut the heart before cryomounting the aortic root.

Figure 2. Atherosclerotic lesion quantification. (**A**) Aortic arch from a 20 weeks old male human *APOB100*-transgenic *Ldlr*$^{-/-}$ (*HuBL*) mouse fed Western diet for ten weeks pinned open and stained for lipid-rich plaques with Sudan IV. Total aortic arch surface area is outlined with the dotted line in white in the micrograph, which also contains a 2 mm scale bar. The dotted lines in yellow outline the total surface area of the brachiocephalic artery. (**B**) Aortic root cross-section at 400 µm from the aortic sinus in a 20 weeks old male *Ldlr*$^{-/-}$ mouse fed Western diet for eight weeks visualized in a micrograph with a 500 µm scale bar. The dotted lines in black outline the total vessel area and atherosclerotic lesions stained with Oil Red O localized in the arterial intima.

Figure 3. Organization of slides for serial sections of the aortic root. During cryosectioning of the aortic root every 10 µm thick section spanning the first 800 µm of the ascending aorta should be collected. A systematic slide organization is needed to obtain suitable sections for various applications. Analysis of lesion composition usually includes Oil Red O staining for lipids and Picrosirius red staining for collagen. Remaining sections are collected and acetone-fixed for immunohistochemistry and immunofluorescence staining. This figure has been modified from Gisterå et al[30].

Figure 4. Atherosclerotic lesions in the aortic root. (**A**) Twenty-eight weeks old male bone marrow chimeras fed Western diet for eight weeks were evaluated to determine the effect of *Smad7*-deficient T cells on atherosclerosis development. Experimental *Ldlr*$^{-/-}$ chimeras received *Cd4-Cre*$^{+}$*Smad7*$^{fl/fl}$ bone marrow and controls received *Cd4-Cre*$^{+}$*Smad7*$^{fl/+}$ bone marrow. The graph shows quantification of atherosclerotic lesion area from eight consecutive sections, 100 – 800 µm from the aortic sinus displayed as lesion fraction of total vessel surface (*Cd4-Cre*$^{+}$*Smad7*$^{fl/+}$*/Ldlr*$^{-/-}$ n=6, *Cd4-Cre*$^{+}$*Smad7*$^{fl/fl}$*/Ldlr*$^{-/-}$ n=9, 2-way ANOVA with Bonferroni's post test, graph shows mean ±SEM, braces indicate significance level for strain comparison). (**B**) The combined dot plot and bar graph shows the mean atherosclerotic lesion area from the aortic root sections (*Cd4-Cre*$^{+}$*Smad7*$^{fl/+}$*/Ldlr*$^{-/-}$ n=6, *Cd4-Cre*$^{+}$*Smad7*$^{fl/fl}$*/Ldlr*$^{-/-}$ n=9, Student's *t*-test) (**C**) Fraction of Oil Red O-stained area in the lesions (*Cd4-Cre*$^{+}$*Smad7*$^{fl/+}$*/Ldlr*$^{-/-}$ n=4, *Cd4-Cre*$^{+}$*Smad7*$^{fl/fl}$*/Ldlr*$^{-/-}$ n=6, Student's *t*-test, ns=non-significant) (**B**-**C**) Dots represent individual mice and bars show mean ±SEM. (**D**) Representative micrographs showing Oil Red O staining (in red color) of neutral lipids in the aortic root 300 µm from aortic sinus (50x magnification) with a 500 µm scale bar. *$p \leq .05$, ***$p \leq .001$. This figure has been modified from Gisterå et al.[31].

Figure 5. Atherosclerotic lesions in aortic arch and brachiocephalic artery. (**A**) Representative en face micrographs of aortic arches with lipid-laden plaques stained with Sudan IV (in orange color) from 20 weeks old mice fed Western diet for ten weeks, visualized together with a 2 mm scale bar. Human *APOB100*-transgenic *Ldlr*$^{-/-}$ (*HuBL*) mice were used as controls and the experimental group consisted of TCR-transgenic mice with LDL-reactive T cells (*BT1*) crossbred to *HuBL* mice. (**B**) Atherosclerotic lesions in the aortic arch (*HuBL* n=10, *BT1xHuBL* n=12; Student's *t*-test). (**C**) Atherosclerotic lesions in the brachiocephalic artery (*HuBL* n=8, *BT1xHuBL* n=9, Student's *t*-test). (**B**-**C**) Dots represent individual mice, bars show mean ±SEM. *$p \leq .05$. This figure has been modified from Gisterå et al.[32].

Supplemental Figure 1. Alternative organization of slides for serial sections of the aortic root. A simplified systematic slide organization for collection of sections from the aortic root. The collection enables Oil Red O staining for lipids and immunohistochemistry or immunofluorescence staining. Dedicated slides for Picrosirius red staining of collagen are omitted.

DISCUSSION:

Cardiovascular disease is the main killer in the world and new preventive measurements are needed[2]. Mouse models of the disease provide a comprehensive platform for investigation of

pathophysiology and experimental treatments[13]. Reliable lesion size quantification is essential for this approach. However, quantification methods differ between laboratories. Standardization and optimization have been an ongoing process since the 1980's[13,27,33,34]. Aortic roots have emerged as the most popular site to quantify experimental atherosclerosis. Cross-sections of plaques enable comparison of plaque volume between groups. En face preparations are favored for lesion quantification in larger segments of the aorta. The en face method visualizes plaque quantity and enables quantification of plaque area coverage, but do not take plaque thickness in account. The biological relevance for observed differences is substantiated by coherent results at different locations in the vascular tree. Evaluating atherosclerosis development at different locations addresses possible site specific effects. The effect of transplanted hematopoietic cells on atherosclerosis development can be assessed in hypercholesterolemic *Ldlr*$^{-/-}$ chimeras. However, whole-body irradiation affects the atherosclerosis process with site specific effects. More prominent atherosclerotic lesions are developed in the aortic root, while reduced lesion development is observed in aortic arches[35].

Importantly, not only lesion size needs to be addressed in studies of experimental atherosclerosis. Lesion composition is also a key parameter. Several plaque features have been associated with manifestations of the disease in humans[36]. Serial sectioning of the aortic root leaves several sections available for careful analysis of plaque composition. Plaque rupture in humans is characterized by a thin fibrous cap with few smooth muscle cells, sparse collagen content and signs of inflammation in the plaques[36]. Although plaque rupture is a rare event in mouse models of atherosclerosis, markers for plaque stability are informative to evaluate. Translational approaches could confirm mechanistic findings from mouse models and uncover important features of human disease[31]. Inflammatory status of atherosclerotic plaques could be determined by immunohistochemistry staining of VCAM-1, MHC class II, macrophages, and lymphocytes[30]. Some protocols use longitudinal sections in the coronal plane of the aortic arch or the brachiocephalic artery for measuring atherosclerotic lesion size and composition[37]. However, this alternative method leaves only few sections to be analyzed, which limits its applications.

An initial critical step in this protocol is the ability to harvest aortas efficiently. Hand-eye coordination under the microscope requires practice and is crucial both for the microdissection and the subsequent pinning of the aortic arch. The next critical step in this protocol is the collection of serial sections from the aortic root. Eighty consecutive sections should be collected for each mouse, which requires both focus and patience. Methodological proficiency could speed up the described processes considerably. Nevertheless, atherosclerotic lesion quantification is still a time-consuming task. New technology, automated handling, and small animal imaging might facilitate quantification of experimental atherosclerosis in the future. The progression of atherosclerosis is slow and most experimental protocols in mouse models take more than four months to complete[13]. Therefore, aortas need to be collected in an optimized way at study endpoints. This protocol provides a comprehensive guide to harvest aortas efficiently and the proposed processing prepares aortas for multi-purpose use including lesion quantification in aortic root, aortic arch, and brachiocephalic artery. Hopefully the protocol can reduce experimental variability, enhance reliability of results, and lead to findings that will pave

the way for new treatments against atherosclerosis.

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DISCLOSURES:

The authors have nothing to disclose.

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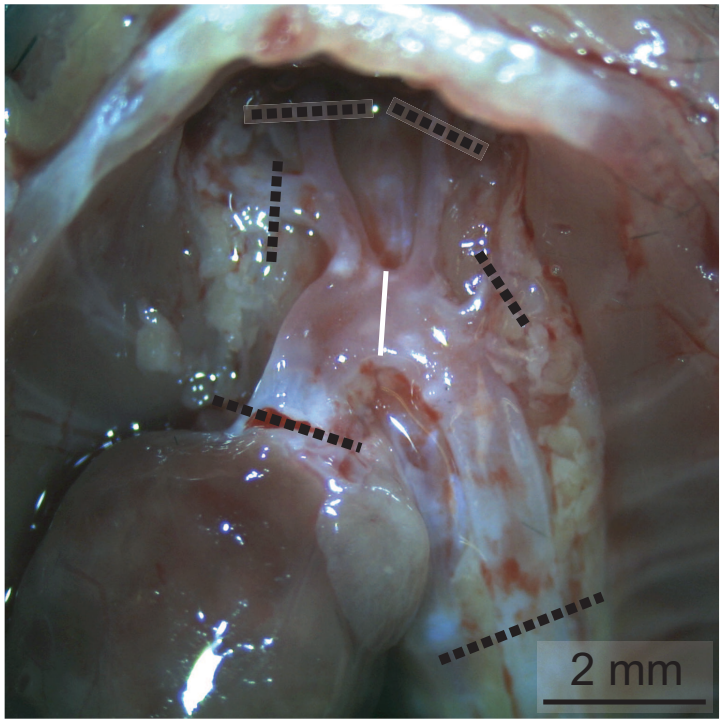
Figure 1

A



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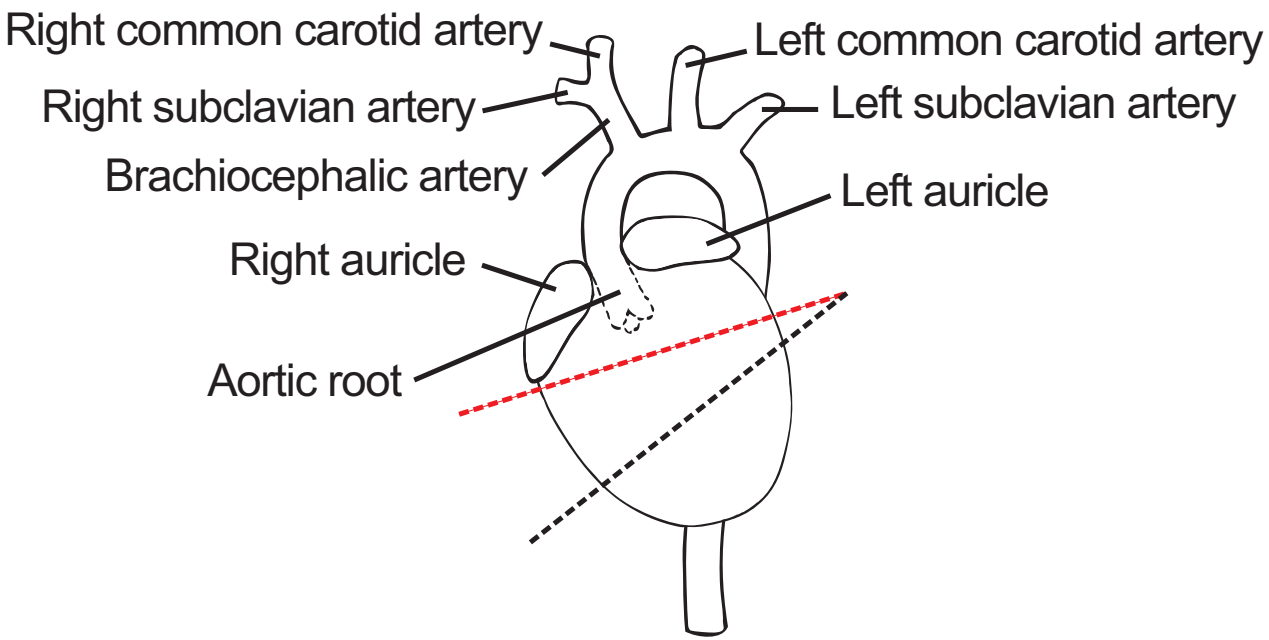
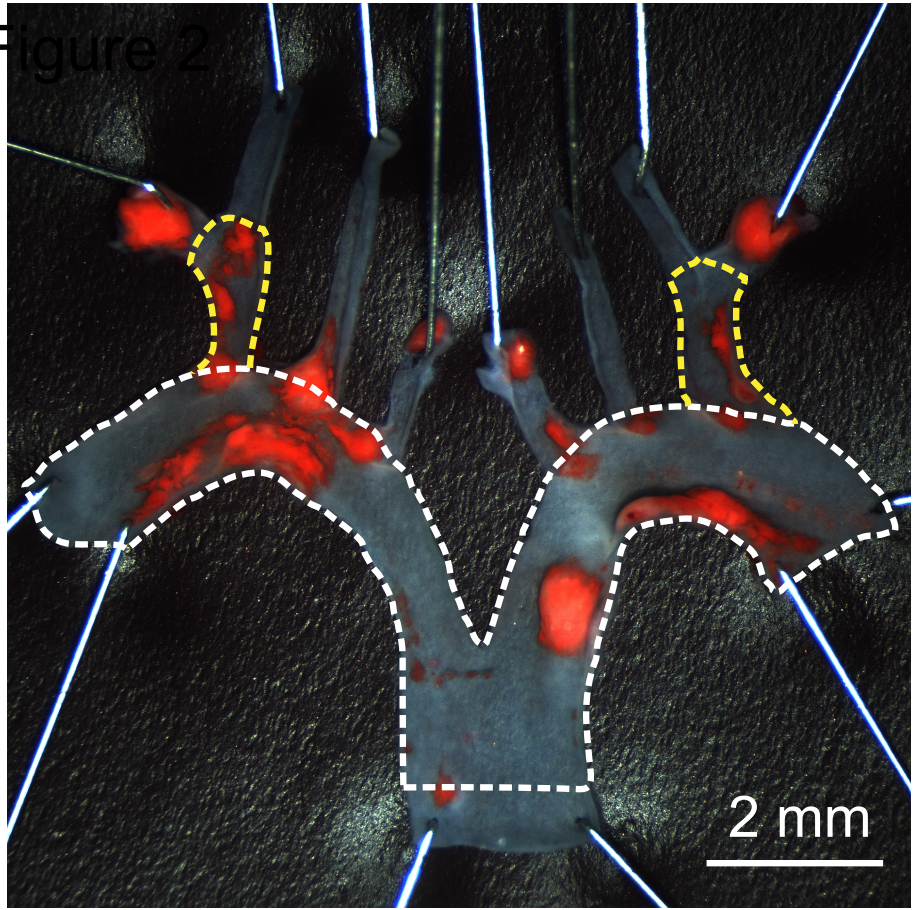


Figure 2



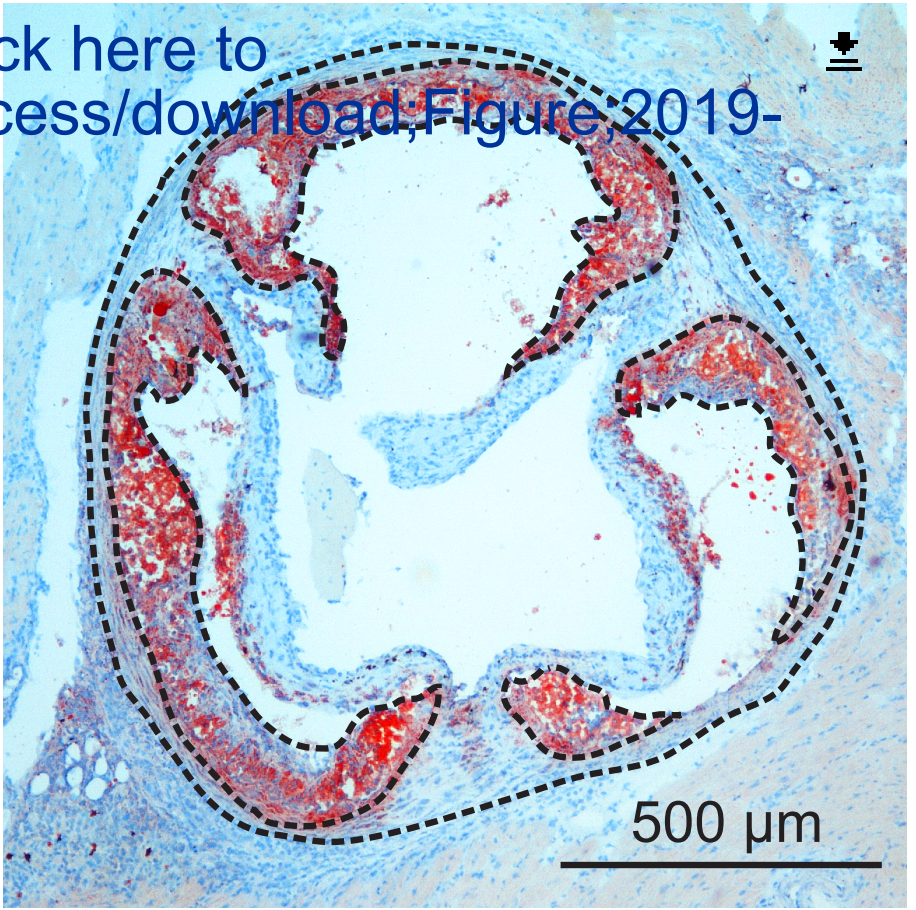
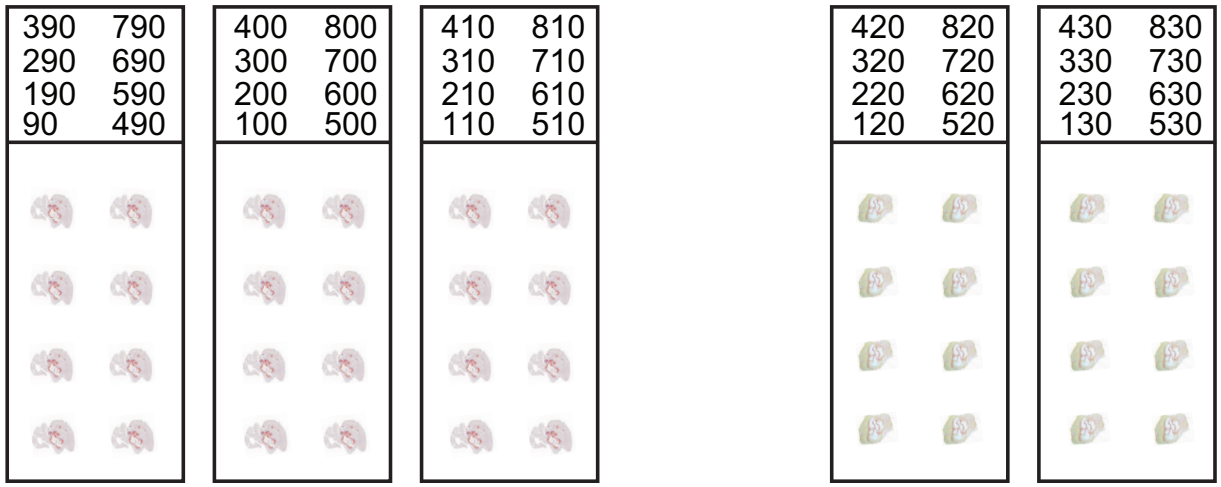
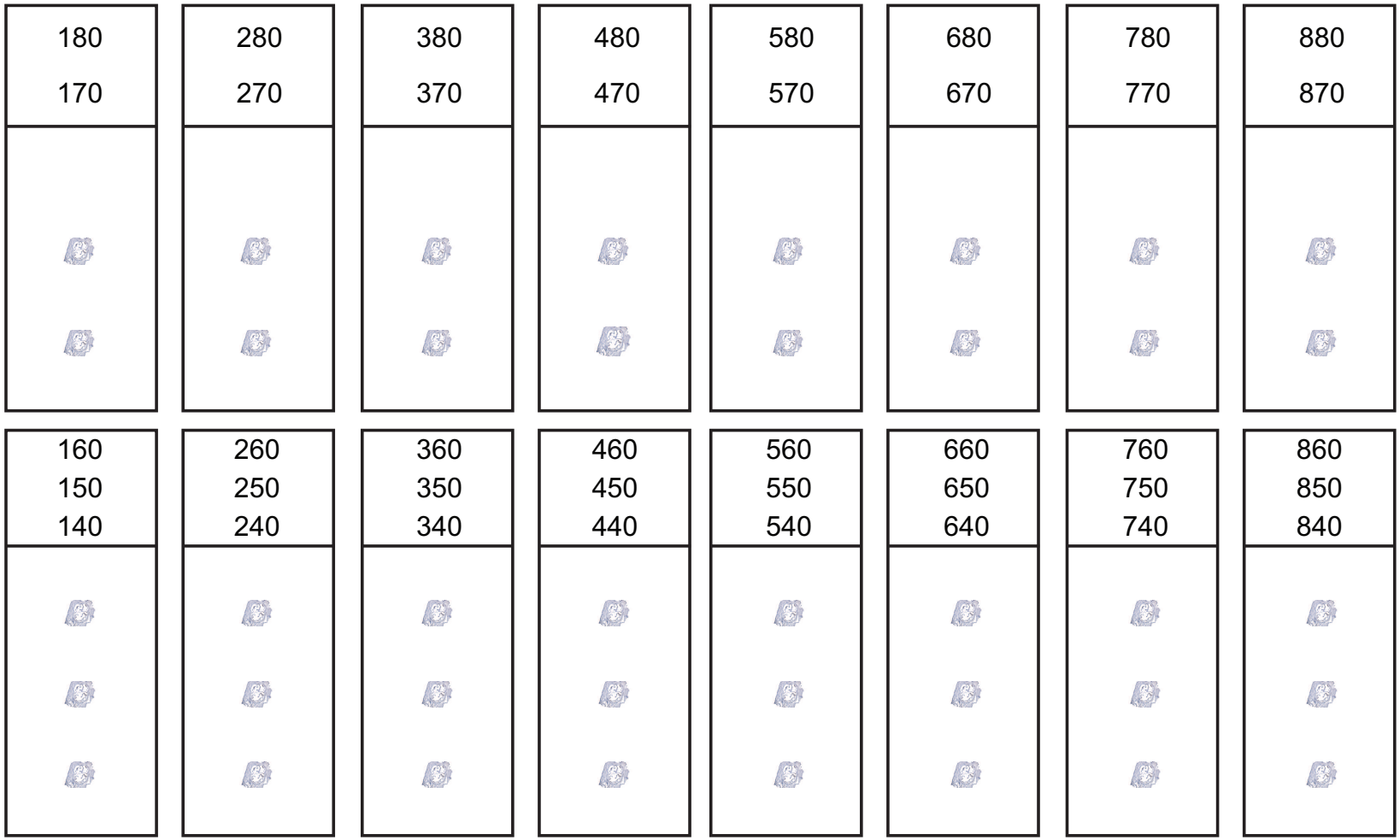


Figure 3

Formaldehyde-fixed slides for Oil Red O (3 slides) and Picrosirius red staining (2 slides)



Acetone-fixed slides for immunohistochemistry staining (16 slides)



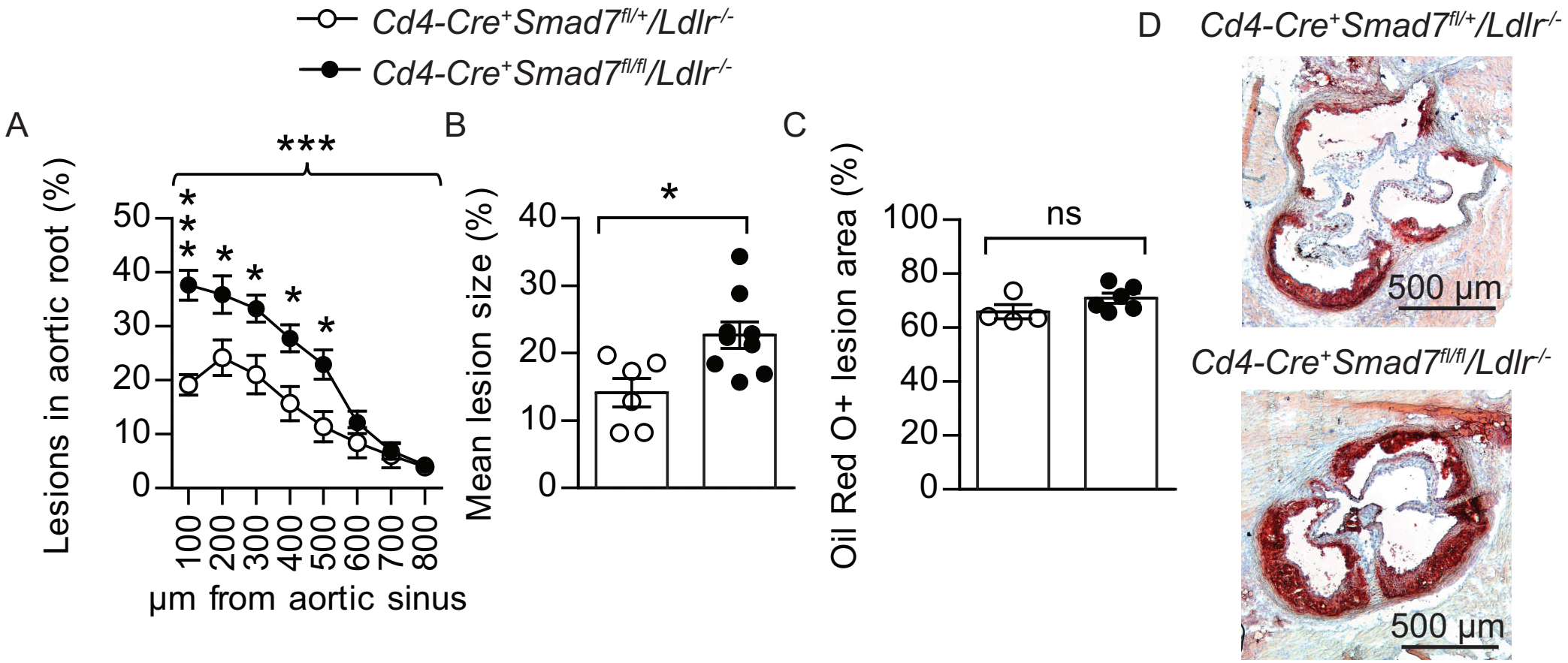


Figure 5

Figure S1

Formaldehyde-fixed slides for Oil Red O staining (8 slides)

160 140 120 100	260 240 220 200	360 340 320 300	460 440 420 400	560 540 520 500	660 640 620 600	760 740 720 700	860 840 820 800
							

Acetone-fixed slides for immunohistochemistry staining (8 slides)

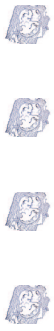
170 150 130 110	270 250 230 210	370 350 330 310	470 450 430 410	570 550 530 510	670 650 630 610	770 750 730 710	870 850 830 810
							

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acetone	VWR Chemicals	20066.296	For fixation of sections for immunohistochemistry.
Black electrical insulation tape (50 mm wide)	Any specialized retailer	-	To create pinning beds for aortic arches.
Centrifuge	Eppendorf	5417C	Benchtop microcentrifuge.
Cork board	Any specialized retailer	-	For cutting hearts in the preparation to cryomount aortic roots.
Cryostat	Thermo Scientific	Microm HM 560	For serial cryosectioning of aortic roots.
Deionized water	-	-	For rinsing and preparation of solutions.
Digital camera	Leica Microsystems	DC480	5.1 megapixel CCD for high-resolution images of aortic arches and aortic root sections.
Dissecting scissors (10 cm, straight)	World Precision Instruments	14393	For general dissection of organs.
Dumont forceps #5 (11 cm, straight)	World Precision Instruments	500341	For microdissection of aorta.
Ethanol 70% (v/v)	VWR Chemicals	83801.290	Highly flammable liquid and vapour, store in a well-ventilated place, and keep cool.
Ethanol absolute ≥99.8%	VWR Chemicals	20821.310	Highly flammable liquid and vapour, store in a well-ventilated place, and keep cool.
Formaldehyde 4% stabilised, buffered (pH 7.0)	VWR Chemicals	9713.1000	Harmful by inhalation, in contact with skin and if swallowed.
ImageJ	NIH	-	Image analysis software.
Iris forceps (10 cm, curved, serrated)	World Precision Instruments	15915-G	Used as anatomical forceps.
Isopropanol	Merck	1096341011	Flammable liquid, causes serious eye irritation, and may cause drowsiness or dizziness.
Kaiser's glycerol gelatine	Merck	1092420100	Aqueous mounting medium containing phenol. Suspected of causing genetic defects.
Light microscope	Leica Microsystems	DM LB2	For analysis during sectioning and documentation of Oil Red O stained micrographs.
Mayer's hematoxylin	Histolab	1820	Non-toxic staining solution without chloral hydrate, but causes serious eye irritation.
Mayo scissors (17 cm, straight)	World Precision Instruments	501751-G	For general dissection.
Micro Castroviejo needle holder (9 cm, straight)	World Precision Instruments	503376	For pinning of aortic arches.
Microcentrifuge tubes	Corning	MCT-175-C	Polypropylene microtubes with snaplock cap.
Microlance 3 needles, 23 gauge	BD	300800	For blood collection.
Microlance 3 needles, 27 gauge	BD	302200	For perfusion of mice.
Microvette 500 µL, K3 EDTA	Sarstedt	20.1341.100	For blood collection.
Microvette 500 µL, Lithium Heparin	Sarstedt	20.1345.100	For blood collection.
Minutien insect pins, 0.10 mm	Fine Science Tools	26002-10	For pinning of aortic arches.
Oil Red O	Sigma-Aldrich	O0625	Not classified as a hazardous substance or mixture.
Optimum cutting temperature (OCT) cryomount	Histolab	45830	For embedding tissue.
Parafilm M	Bemis	PM992	Paraffin wax film used to create pinning beds for aortic arches.
Petri dishes (100x20 mm)	Any cell culture supplier	-	Proposed as a storage container for pinned aortas.
Phosphate buffered saline (PBS)	-	-	Sterile and RNase-free solution is required for perfusion of mice.
Qualitative filter paper (grade 1001)	Munktell	120006	For filtering Oil Red O working solution (typical retention 2-3 µm).
RNA*later* RNA stabilization reagent	Qiagen	76106	For stabilization of RNA in tissue samples
RNase*Zap* RNase Decontamination Solution	Invitrogen	AM9780	A surface decontamination solution that destroys RNases on contact.
Scalpel handle #3 (13 cm)	World Precision Instruments	500236	For cutting hearts in the preparation to cryomount aortic roots.
Standard scalpel blade #10	World Precision Instruments	500239	For cutting hearts in the preparation to cryomount aortic roots.
Stereomicroscope	Leica Microsystems	MZ6	For dissection and en face documentation
Sudan IV	Sigma-Aldrich	S4261	Not classified as a hazardous substance or mixture.
Superfrost Plus microscope slides	Thermo Scientific	J1800AMNZ	To collect aortic root sections.
Tissue forceps (15 cm)	World Precision Instruments	501741-G	For general dissection.
Tissue-Tek cryomolds (10x10x5 mm)	Sakura	4565	For embedding aortic roots in OCT.
Vannas scissors (8 cm, straight)	World Precision Instruments	503378	For microdissection of aorta.

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Centa et al.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

AU: We thank the Editor for this possibility. We have updated the manuscript using track changes.

2. Please revise lines 49-52 and 58-61 to avoid previously published text.

AU: These lines have been changed in the updated manuscript.

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AU: The files are now uploaded with the revised manuscript. The permissions have been obtained in accordance with correspondence with dr Upponi at JoVE.

4. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

AU: We have removed personal pronouns from the revised manuscript, except from the acknowledgment section.

5. Please replace commercial language (parafilm) with generic terms.

AU: We have now changed parafilm to “paraffin wax film”.

6. 1.2: Please specify the age, gender and type/strain of mouse.

AU: The age, gender and mouse strain are now stated in the figure legends.

7. 1.9: Please describe how to clean the instruments.

AU: The cleaning steps have been included in the revised manuscript.

8. 1.10: Please specify “a long time”.

AU: This has been specified in the revised manuscript.

9. 1.12: Are plasma and serum transferred to separate tubes? Please specify.

AU: Correct, this is now clarified.

10. 2.8: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g., button clicks or menu selections for software actions, numerical values for settings, etc.).

AU: This is now included in the revised manuscript.

11. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

AU: These changes have been made in the revised manuscript.

12. Please ensure that the highlighted content should be continuous and contain essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

AU: The text highlighted in yellow describes cryomounting the aortic roots, cryosectioning, and en face analysis of the aortic arch and brachiocephalic artery. We think protocol steps 1.14-1.16, 2.1-2.7, and 3.1-3.5 are the most crucial for the story and would like to propose those to be visualized in the video.

13. Figure 2: Please include a scale bar at the lower right corner for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

AU: This has been included in the revised figures.

14. References: Please do not abbreviate journal titles.

AU: This has been corrected.

Centa et al.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Centa et. al. provides a technical description for the evaluation of atherosclerosis in aortic roots and the aortic arch in mice under a Western-type diet. The protocol describes a point by point en face preparation and Sudan IV staining of the aortic arch and a method to perform cross-sections of the aortic root and oil-red O staining with frozen samples.

Major Concerns:
1. Although mentioned in the abstract and introduction, the article does not offer a technical description or protocol to quantify atherosclerosis in the brachiocephalic artery. So, either this has to be removed from the text or authors should offer a detailed description too.

AU: We have chosen to expand the description of en face analysis of the brachiocephalic artery in the revised manuscript. Protocol section 1.9-1.11 describes the harvesting procedure of the brachiocephalic artery. We have emphasized the pinning of the brachiocephalic artery in section 2.3 in the revised manuscript and changed the title for section 2 in the protocol. The visual display in Figure 2B explains the lesion analysis in the brachiocephalic artery further. The image analysis strategy to quantify lesions in the brachiocephalic artery is expanded in the revised manuscript. The protocol step 2.9 has now three additional substeps to explain the quantification. We have published results based on this brachiocephalic lesion analysis method last year in Circulation[1] and the current manuscript provides one example of the results from this publication in Figure 5C. However, en face analysis does not provide information of plaque composition and volume, which potentially could be of importance in some studies. As we explain in the discussion, alternative ways of analyzing the brachiocephalic artery exist, and we have updated the revised manuscript with a reference to such analysis (new ref 37).

2. The title of the article is too general. A more specific title (e.g. including aortic root and aortic arch) could help scientists to search this article and could give a better idea of the scope of the protocol.

AU: The summary and abstract provide details for the potential reader about the specific sites for quantification of atherosclerosis that the protocol describes. Specific queries for these sites should be picked up by most search engines and direct readers to our manuscript. The methodology described in the manuscript has been developed in Göran Hansson's laboratory over the past quarter-century and has been well-perceived by researchers in vascular inflammation. To reach a wider audience, we would like to keep the title short. If needed, we can construct a new title in consultation with the Editor to optimize crawlability.

3. Line 111. The authors stated: "there are no fully standardized protocols to quantify atherosclerosis in mouse models of atherosclerosis". The American Heart Association recently published a scientific statement detailing recommendations for proper experimental design, procedural standardization, analysis, and reporting of animal atherosclerosis studies (Daugherty A. et al. ATVB 2017). This article highlights the benefits and limitations of the predominant techniques used in atherosclerosis studies. For example, en face Sudan IV staining of the aorta is often performed as first read-out in atherosclerosis studies. Although en face Sudan IV staining of lipid deposition is a good method for assessment of global plaque burden, it is unable to distinguish early-stage fatty streak lesions from more advanced late-stage lesions. As such, the interpretation of en face staining is often ambiguous

and superficial. The authors should include this reference and carefully explain the advantages and limitations of their protocol accordingly.

AU: We thank the reviewer for pointing out this careless phrase of ours. We have changed it in the revised manuscript. When reading experimental atherosclerosis reports the variability in quantification methodology and data presentation is surprisingly large. One of the more frequent requests that we receive from other researchers is to share a detailed protocol regarding our method for lesion quantification. In our present manuscript, we provide a very detailed presentation of lesion quantification, which has been developed in Göran Hansson's laboratory over the past 25 years. With the JoVE format that allows video visualization of critical steps we think that the manuscript will be a valuable resource for researchers that are setting up new mouse atherosclerosis experiments. Compared to other protocols, we have added many tips and tricks that we think are especially valuable. The described methods in the manuscript are in line with the 2017 ATVB statement from Daugherty A. et al. We have now incorporated the reference in the revised manuscript (new ref 26). We agree with the reviewer that en face analysis is a good method for lesion size analysis, which is often used as a first read-out since it is relatively fast. This is also pointed out by the 2003 paper by Daugherty A. et al that our manuscript refers to. In our hands, lesion size evaluation is usually more straight-forward and less ambiguous than lesion composition analysis. With that said, we emphasize the immense importance of lesion composition analysis in the discussion section. The discussion has been updated with the limitations of en face analysis.

4. Both the introduction and the discussion are a little long. The authors should focus on the discussion to enumerate the advantages of using this method to quantify atherosclerosis in the aortic root and aortic arch compare to others.

AU: We have adhered to journal guidelines regarding the length of introduction and discussion. As pointed out in the previous point, we have made changes in both these sections in the revised manuscript to address the reviewers' points.

Minor Concerns:
5. Please state how long the APOB100-transgenic Ldl-/- mice were on a western diet?

AU: This is now included in the figure legend.

6. Note in line 158: The authors should clearly mention that NOT paraformaldehyde perfusion-fixation of tissue is an optional step.

AU: This has been further emphasized.

Reviewer #2:
Manuscript Summary:
The manuscript outlines the analysis of atherosclerosis in the aortas of hyperlipidemic mice. It is very informative and detailed and I have nothing to add or to correct. Well done.

AU: We thank the reviewer for this comment.

Reviewer #3:
Manuscript Summary:
The author well described the introduction and background of atherosclerosis. Overall protocol is well-written even though animal dissection and tissue sectioning are hard to describe. Some of the part hard to follow without picture or video. This protocol will be a good reference to study atherosclerosis.

AU: We thank the reviewer for this comment. The accompanied video production for the manuscript will be very valuable to describe the protocol steps further. Since several previously published protocols lack some useful details, we wanted to provide an as detailed description as possible of all steps, even though some of them are difficult to explain in writing.

Major Concerns:
The manuscript does not contain the benefit of this protocol over other published methods. Since this paper is a method paper, authors need to introduce more about the other method and compare to this protocol especially in paragraph 8 in the introduction. This can be discussed in the Discussion part. And in the same paragraph, the authors said 'Lack of standardization may cause observed differences in disease development.', So far the other literature are using Oil Red O or Susan IV and en face/ frozen section are the standard method for atherosclerosis study, please explain what is the lack of standardization mean in paragraph 8 and how the authors approached data to make standard protocol than other publications.

AU: We thank the reviewer for pointing this out. A similar point was raised by reviewer 1 in question 3. Please see our response to this question. In summary, we have changed the cited phrase and improved the introduction and discussion. We receive many requests for detailed protocols regarding lesion quantification, which motivated us to put together this protocol that has been under optimization in Göran Hansson's laboratory for the past quarter-century. We hope that the JoVE format will help explain hard-to-grasp steps and benefit researchers that are setting up new mouse atherosclerosis experiments.

In Figure 4B, C, and D, Figure B were measured by Oil Red O stained area, but it is not clear why Oil Red O+ lesion area has no significance in Figure 4C, and then Figure D Oil Red O stain images have a significant difference.

AU: Figure 4B measures lesion average lesion size according to the protocol step 4.7 in the revised manuscript. Figure 4C measures Oil Red O stained lesion area according to protocol step 4.8. This is clarified in the figure legend. Figure 4D shows representative micrographs of aortic root sections. The experimental mouse has larger lesion (quantified in 4A-B), but there are no differences in fraction of Oil Red O stained area in the lesions (quantified in 4C).

In Figure 5A, representative picture do not seem to have difference in the atherosclerotic lesion in the brachiocephalic artery.

AU: This is a good point and alert observation. The pictures were selected to represent the observed difference in aortic arch lesions, which is stated in the figure legend. The observation highlights the importance of sample size and quantitative assessment of lesions in more than one vascular bed, which is discussed in the manuscript.

Minor Concerns:
In agreement of other literature, aortic root, arch, and brachiocephalic area are the most preferred lesion area for atherosclerosis, but thoracic and abdominal aortas area is still missing. Those area might have bigger lesion than brachiocephalic area.

AU: Our protocol does not quantify atherosclerosis in thoracic or abdominal aorta, but saves these parts for other applications, such as RNA or protein analysis. We agree that the protocol needs to be adjusted according to specific research questions, which is mentioned in the manuscript introduction. We have taken many aspects in consideration to develop this protocol and to make the most use of every single mouse we sacrifice. Generally, lesions initially develop in ascending aorta and the aortic arch region. Measurable lesions in descending thoracic and abdominal aorta are only present in animals with more advance disease[2]. Hence, we find other applications than quantification more suitable in many experiments for these parts. We have updated the representative result section to clarify this.

In protocol 1.13, please draw the line 30 degree angle in Figure 1B, it will help understand better for the readers.

AU: Figure 1B has been updated and we have highlighted this step to be explained in the video production. We have also updated the protocol text to be more accurate.

Protocol 2.7, please define the metal weighs, especially size. that seems should fit in a petri dish.

AU: They are 10x20x5 mm and weighs 15 grams, but anything that counteracts the buoyancy of the parafilm bed will do.

Please Specify the distance from the left subclavian artery branch to cutting line.

AU: Since we do not quantify atherosclerosis here, the cutting line does not have to be that exact. We usually cut it 1.5 mm from the aorta, as is displayed in Figure 1A.

Protocol 1.12, are the blood collection tubes from protocol 1.3? if so, please mention that.

AU: Yes, this has been clarified in the revised manuscript.

References

1 Gistera, A. *et al.* Low-Density Lipoprotein-Reactive T Cells Regulate Plasma Cholesterol Levels and Development of Atherosclerosis in Humanized Hypercholesterolemic Mice. *Circulation.* **138** (22), 2513-2526, (2018).

2 Daugherty, A. *et al.* Recommendation on Design, Execution, and Reporting of Animal Atherosclerosis Studies: A Scientific Statement From the American Heart Association. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **37** (9), e131-e157, (2017).

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