# Supplementary material for:

# Synchrotron-based visualization and segmentation of elastic lamellae in the mouse carotid artery during quasi-static pressure inflation

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## Methods

### *2.3 Image post-processing: automatic 2D segmentation of lamellae – detailed description*

During post-processing the images underwent a fully automated sequence of post-processing steps that were part of a segmentation algorithm (Supplementary Figure S1) that had been specifically designed to extract individual elastic lamellae from the images (Figures 2 and 3). All commands can be found in the image processing toolbox in Matlab R2016b (The Mathworks, USA).

The first intermediate goal was to set all pixels in– and outside the vessel wall to zero. To that end the original image (Figure 2a) was first binarized from greyscale to black and white. Since different scans can have different levels of background noise, we increased the value of the binary threshold (i.e. the greyscale level that serves as a cut-off value to bin pixels into a value of either 0 or 1) in an iterative for-loop from 0 to 1 in steps of 0.01. The number of white pixels (binned into a value of 1) corresponding to each binary threshold was calculated and stored in an array. The optimal binary threshold was chosen as the threshold corresponding to the first index that was larger than the index corresponding to the maximum and for which the second derivative fell below a pre-defined value of 100 (final result of this iterative process: Figure 2b). We found this indirect approach to be more robust than a directly hardcoded threshold, because the binary threshold that was obtained in this way corresponded to the binary image that had the most information (i.e., white pixels belonging to the wall) and the least possible noise (i.e., white pixels belonging to the background). Connected items smaller than 10 pixels were subsequently removed from the binary image and the image was morphologically closed such that the largest remaining connected item in the image was the vessel wall (Figure 2c). Since at this stage we were only interested in the outer contour, a succession of dilate and erode operations was used to remove erroneous spikes protruding from the vessel wall and a convex hull was computed (Figure 2d). Once the outer convex hull had been identified, its inverse could be subtracted from the original image to eliminate background noise (Figure 2e). A series of Boolean operations was then executed to identify the pixels belonging to the arterial lumen. The inverse of the outer hull (i.e., an image where all pixels outside the vessel were set to 1) was added to the original binary image and the resulting image was subsequently inverted, leaving the inner lumen as the largest single connected item. After filling holes in this image (Figure 2f), the best fitting filled circle was computed, shrunk with 2 pixels, and added to the image in order to compensate for erroneous inward-oriented "leaks" (Figure 2g). The (inverted) outer hull and the inner convex hull were subsequently subtracted from the original greyscale image (Figure 2h).

The second intermediate goal was to process the greyscale image such that it represents the connectivity of the lamellae. Since the goal of this step was to find the inner lamella (see further), the outer layers had no contribution in the post-processing. In order to expedite the process we therefore first removed the outer half of the aorta (Figure 3b), based on the information that was obtained during image pre-processing (outer convex hull in Figure 2d and approximate wall thickness in Figure 2c). Once the input image was determined, we set out to find the optimal binary threshold (Figure 3c). Similar to what was done during image pre-processing (Figure 2b), the binary threshold was increased in an iterative for-loop from 0 to 1 in steps of 0.01. For every binary threshold the corresponding image was skeletonized such that each meaningful connection in the resulting graph structure was represented by a single line only, and the number of branch points was calculated and stored in an array. The optimal threshold was chosen as the value corresponding to the lowest binary threshold at which both the first and second derivative of the branch array were negative, which is equivalent to a local maximum in the number of valuable connections within the structure (final result of this iterative process: Figure 3d). Edge gaps with a gap size of 3-5 pixels were filled in (Figure 3d, green arrows) and loose edges (i.e. edges of which either one or both endpoints were not connected to any other edge) were removed (Figure 3d, yellow arrows).

The third intermediate goal was to find the paths that represent the elastic lamellae. We first calculated the coordinates ($x_c$, $y_c$) of the geometric center of gravity of the outer convex hull. All pixels along the vertical axis ($x=x_c$, $y>y_c$) were subsequently set to 0 (Figure 4e, red arrows). Then, a connected graph was calculated in which each elastin line on the image corresponded to an edge in the graph (Figure 3f). The connectivity between these edges was stored in a sparse bi-directional matrix, with two junctions for each intersection between two edges (one junction for each direction). In the next step the starting and ending points of each of the three lamellae were defined as the edges closest to the geometric center that intersected with the pixel column left and right of the zero-column, respectively (figure 3f, bottom). For each lamella the shortest path along the graph from its start to its end point was then calculated as follows. First, the inner convex hull was eroded with 10 pixels and the outer boundary of the eroded hull was calculated. Then for every edge in the graph the distance to the eroded hull was obtained (taking the mean value for all points along that edge, Figure 3g). We used the eroded hull rather than the geometric center of gravity to calculate these distances because it makes the algorithm more robust in low-pressure cases where the artery is buckled. To every junction in the sparse matrix a weight was then attributed that corresponded to the average distance to the eroded hull of both edges belonging to that junction. These weights were subsequently used in a modified version of Dijkstra`s shortest path algorithm(1) (Supplementary Figure S1c). The original Dijkstra algorithm (Figure S1d) would define the 'shortest path' as the path between starting edge and ending edge with the lowest total accumulated weight along its junctions(1). When a detour via the second lamella is, however, shorter (i.e., contains less edges) than the path via the first lamella, Dijkstra`s algorithm will always prefer that path, even if the individual weights of junctions along the inner lamella are lower. That is why we modified Dijkstra`s algorithm such that it no longer yields the path with the lowest total accumulated weight but the path with the lowest averaged weight per junction (see illustrations at bottom of Figure S1c and S1d). The shortest path according to this modified Dijkstra algorithm (Figure 3h) was subsequently back-projected onto the original image (Figure 3i). As a last step, the path was filtered in order to correct for discrepancies that were introduced during the skeletonization step (e.g. the transition from Figure 3c to Figure 3d). For each pixel, the local lamellar thickness was calculated as twice the distance between the curve representing the segmented lamella (corresponding to the local centerline of the lamella, Figure 3i) and the inner lining of the binarized image (corresponding to the local inner curvature of the lamella, innermost pixels of Figure 3c). This local thickness array was used to smooth the lamellar path (Figure 3j). First an overall target thickness was calculated in order to reduce the influence of local image artefacts. Then all thickness values greater than the mean thickness were thresholded to the mean thickness in an iterative loop, which was repeated until the ratio of standard deviation to mean thickness was smaller than a predefined value of 20%. The mean thickness at the end of this iterative smoothing process was defined as the target thickness. Finally, all pixels on the lamella centerline for which the local distance to the inner curvature was greater than half the target thickness were pushed inward (i.e. toward the barycenter), such that the new distance to the inner curvature was equal to half the target thickness (Figure 3j). Once the lamella centerline and corresponding local thickness array had thus been calculated, this information was used to remove the segmented lamella from the original image. This yielded a new input image similar to the one shown in Figure 3b, but now with the second lamella being the innermost lamella (Figure 3k). Steps b to j were repeated, resulting in a segmented second (middle) lamella (Figure 3l). The second lamella was then removed from the original image (Figure 3m) and the outermost lamella was segmented (Figure 3n). Finally the outer lining of the adventitia was segmented from the outer convex hull. The node-to-node distance from the adventitia to the centerline of the outer lamella was calculated. All points on the adventitia that were located further from the outer lamella than the mean distance plus two standard deviations were considered to be image artifacts, and pushed inward such that the distance was equal to the mean distance. In a final step, the resulting adventitial lining was smoothed with a Savitsky-Golay filter and all segmented layers (outer lining of adventitia and centerline of three lamellae) were back-projected onto the original image (Figure 3o).

***Statistical analysis: ApoE$^{-/-}$ vs WT mice***

Our dataset consisted of n=6 WT mice and n=6 ApoE $^{-/-}$ mice. Differences between ApoE$^{-/-}$ and WT mice were analyzed at each pressure level using a two-sided student`s t-test. Tests were carried out on mean values, i.e. n=6 data points for each of the 18 combinations of genetic background and pressure level, where each data point represents the mean value of n=75 segmented images from that scan (with the exception of images that had been discarded). A p-value of 0.05 was considered significant (*).

## Results and Discussion

### *Comparison between ApoE $^{-/-}$ and WT mice*

Earlier findings by our (2) and other (3) groups have reported an increased aortic stiffness in ApoE$^{-/-}$ mice, which is believed to be one of the factors leading to the increased susceptibility of these mice to plaque formation (4). Here, we did not find any statistically significant difference in diameter (Figure S2 a-c), straightness (Figure S2 d-f) or lamellar length (Figure S2 g-i) between ApoE $^{-/-}$ and WT mice at any pressure level. This is in line with recent publications, which have shown that there is no difference in carotid artery stiffness between ApoE$^{-/-}$ and WT mice (5, 6).

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